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PROTOPLAST DEVELOPMENT, REGENERATION AND  
FUSION IN Brevibacterium lactofermentum

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PROTOPLAST DEVELOPMENT, REGENERATION AND  
FUSION IN Brevibacterium lactofermentum

By

Susanne E. Keller

A DISSERTATION

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## INTRODUCTION

Although microorganisms are currently known to produce hundreds of substances of commercial value, relatively few are produced for sale (Eucheigh, 1981). This is due primarily to the lower cost and higher yield of chemical synthesis as compared to biological fermentation. In microorganisms, biological pathways to the production of various industrial chemicals are often subject to strict control mechanisms which prevent their overproduction in the wild type. Production, therefore, depends upon the ability to overcome these controls either by often costly chemical manipulations or the development of such mutants lacking such control mechanisms. Traditionally, the development of mutants was dependent of the screening of enormous numbers of artificially created mutants, a technique that was at best slow and laborious. With the advent of new genetic methods such as recombinant-DNA techniques, direct manipulation of biological pathways within microorganisms becomes a possibility. The creation of such new microorganisms makes fermentation of various economically important substances a very attractive proposition.

However, the creation of new industrially useful strains of microorganisms requires that basic research provide us with the knowledge necessary to take advantage of new genetic methods. For example, in both E. coli and B. subtilis, various methods of genetic transfer are well established. A genetic map and various methods of mapping of the genomes of these organisms is also readily available. This information is vital to the success of newer methods of genetic engineering. Unfortunately, this is not the case with all industrially important microorganisms. One such genus of industrial importance for which these methods have not been well established is Brevibacterium. It was the purpose of this work to establish a reliable method of genetic transfer and a genetic map of Brevibacterium, using B. lactofermentum as the model strain.

The uses and economic importance of the genus Brevibacterium are wide and varied. Expansion of its use and improvement of strains for commercial use would be of considerable economic interest. As mentioned such development currently depends on traditional development and screening of enormous numbers of mutants. Reliable methods for genetic transfer and genetic mapping of this species would enhance genetic improvement of these industrially important strains of Brevibacterium.

## LITERATURE REVIEW

### Uses and Economic Importance of *Brevibacterium*

One bacterial genus of considerable industrial importance is *Brevibacterium*. For example, *B. flavum* is one organism used commercially to produce glutamic acid or monosodium glutamate (MSG) for use as a flavoring agent. Approximately 300,000 tons of MSG are produced per year. This represents sales of 1,080 million dollars per year (Eucleigh, 1981). A number of other species of *Brevibacterium* are also considered glutamic acid producers. These species include *B. divaricatum*, *B. aminogenes*, *B. lactofermentum*, *B. saccharolyticum*, *B. roseum*, *B. ammoniagens*, *B. alamicum*, and *B. thiogentitalis* (Abe and Takayama, 1972). In addition to the more traditional approach used to produce MSG by *B. flavum*, a novel method using temperature-sensitive mutants of *B. lactofermentum* was developed by Momose and Takagi (1978). Normally, glutamate production by *Brevibacterium* requires that the media be biotin deficient or that penicillin or surface active agents such as Tween 60 are added to the media. The mutant developed by Momose and Takagi (1978) was found to produce glutamic

acid in biotin-rich media without the presence of penicillin or surface active agents when the temperature was raised from permissive (30°) to non-permissive (37°).

Along with glutamic acid, B. flavum can be manipulated to produce lysine, an essential amino acid. Currently 80% of lysine is produced by fermentation (Eucleigh, 1981). Other amino acids which have been reported to be produced by various members of the genus Brevibacterium include alanine, valine, homoserine, o-ethylhomoserine, o-propylhomoserine, phenylalanine, threonine, proline, tyrosine, aspartate, histidine, serine, isoleucine, and leucine (Abe and Takayama, 1972; Ikeda et al., 1976; Nakamori and Isamu, 1970; Shiio et al., 1982; Tsuchidu et al., 1975a; Tsuchidu et al., 1975b; Yoshinaga, 1969). Although not all these are economically viable fermentations, the variety serves to illustrate the point that, with new genetic techniques, the industrial fermentation of any amino acid by Brevibacterium may be possible in the near future.

In addition to the production of amino acids, this genus is important in the production of nucleotides and nucleotide-related products such as 5'-inosinic acid (Nara et al., 1969; Sato and Furuya, 1977; Teshiba and Furuya, 1982). Inosinic acid is an important flavor-enhancing agent of foods and of considerable economic significance. The species used in its production is B. ammoniagenes. Coenzyme A is also produced via biological

fermentation utilizing B. ammoniagenes (Asada et al., 1982; Shimizu et al., 1979).

Another important use of Brevibacterium in the food industry is the production of various cheeses (Rose, 1981). B. linens in mixed cultures usually with Streptococcus lactis and S. cremoris is involved in the production of limburger, brick, muenster, trappist, port and duSalut cheeses (Rose, 1981; Schmidt et al., 1976). Its primary role appears to be in flavor development.

Uses of Brevibacterium discussed to this point have centered on those already of considerable economic importance. A survey of literature involving Brevibacterium indicates other possible roles for this genus, including biodegradation of herbicides (Horvath, 1971; Rott et al., 1979). Some aid the metabolism of hydrocarbons (Atlas and Bartha, 1972; Pirnik et al., 1979). This latter ability may make them important in biodegradation of hazardous pollutants. Another possible use of Brevibacterium, the production of feed protein from mesquite wood, has been investigated by Fu and Thayer (1975). Mesquite wood itself is not useful as feed and its conversion to protein would render those areas where only mesquite wood grows as economically productive. One final use for Brevibacterium may be in the isolation and use of novel endonucleases for use in characterization of DNA or in recombinant DNA work. A few such nucleases have already been characterized

(Basnak'yan et al., 1981; Gerlinas et al., 1977).

### Classification and Taxonomy of *Brevibacterium*

Brevibacterium as a group is rather complex and heterogeneous. In the 8th edition of Bergey's Manual it is placed within the coryneform group of bacteria Genera incertae sedis. Attempts to clarify the position of Brevibacterium have been made by numerous methods using different criteria (Bergey & Manual, 8th Ed., 1974; Collins et al., 1979; Crombach, 1971; Jones, 1975; Keddle and Cure, 1979; Komagata et al., 1969; Stackebrandt and Fiedler, 1975; Seiler, 1983; Yamada et al., 1976; Yamada and Komagata, 1970) and with variable success. No attempt will be made here to reclassify or clarify the taxonomy of Brevibacterium. However, certain characteristics examined by researchers and unique to this group will be discussed.

General characteristics of Brevibacterium are similar to those of Corynebacterium and Arthrobacter. They have considerable morphological diversity but typically appear as short, aerobic, non-sporulating, gram-positive rods (Bergey's Manual, 8th Ed., 1974). Yamada and Komagata (1970) attempted to characterize Brevibacterium as well as the other related groups of coryneform bacteria on the basis of type of cell division, cell wall amino acid content and DNA base composition. None of the factors discussed could be utilized to distinguish Brevibacterium from other

coryneform bacteria. GC content was found to vary considerably (46.6-70.5). This is supported by latter studies done by Pitcher (1983). Cell wall content varied but remained consistent with variation found in Corynebacterium and Arthrobacter. It is interesting to note that glutamic acid producers, regardless of genus, had a narrow range of GC content, similar cell wall amino acid content, and a single mode of division. Examinations by Keddie and Cure (1979) of cell wall composition of coryneform bacteria also found wide diversity in Brevibacterium in general. However, glutamic acid producers, regardless of genus, appeared similar. Such strains all contained DL-DAP along with the sugars, arabinose and galactose. Thus these glutamic acid producing strains among different genera would seem, therefore, to be more related to each other than individual strains within the grouping Brevibacterium.

Other researchers have attempted to use the unusual menaquinones found in coryneform bacteria as a classification method (Collins et al., 1979; Yamada et al., 1976). Coryneform bacteria were found to contain the same type of menaquinone, referred to as MK-9 (H<sub>2</sub>). Because this is the same type found in Corynebacterium bovis and also on the basis of similar mycolic acids within glutamic acid producers and true corynebacteria, Collins et al. (1979) recommended the transfer of all glutamic acid-producing strains they tested to Corynebacterium-sensu stricto. These researchers

also point out that their data support the hypothesis that glutamic acid producers should in fact be reduced to synonymy with C. glutamicum. This hypothesis is further supported by the numerical taxonomic studies of Seiler (1983). One method to resolve the difficulty in classification of Brevibacterium within Corynebacterium may be found in DNA-DNA or DNA-RNA homology studies. Such studies could provide more conclusive evidence on the relationship of the various genus and species to each other. Stackebrandt and Fiedler (1975) have done one such study. Since, the number of organisms used within corynebacteria in general and specifically Brevibacterium are very small and of little use in classification, additional work in this area would be beneficial.

#### Protoplast Development and Fusion

Transformation, transduction, or conjugation have never been reported for Brevibacterium. However, protoplast fusion has been reported for B. flavus, B. lactofermentum and C. glutamicum, and therefore, holds promise as a method of genetic exchange (Ajinomoto Co., 1983a, 1983b). Optimal conditions have not yet been well established.

Protoplast fusion has been used for genetic studies in a large number of organisms (Ferenezy, 1981; Hopwood, 1981). With the advent of the use of PEG as a fusogen by Koa and co-workers with plant protoplasts, various procedures for

fusion have been developed for both procaryotes and eucaryotes (Baltz, 1978; Fodor and Alfold, 1979; Hales, 1977; Kao and Michayluk, 1969; Kao et al., 1974; Pontecorvo, 1975; Schaeffer et al., 1976). Proper use of protoplast fusion as a genetic technique depends upon the ability to develop and revert protoplasts. The method used depends upon the type of organism in question. Within the gram-positive procaryotes such as B. subtilus, B. lichenformis, and B. megaterium, which have been extensively studied, a simple lysozyme treatment was sufficient to produce protoplasts (Bugaichuk et al., 1981; Fodor and Alfoldi, 1976; Schaeffer et al., 1976; Wyrick and Rogers, 1973). In the case of B. brevis, achromopeptidase was used successfully to produce protoplasts. Resistance to lysozyme of this B. brevis strain was attributed to a thick protein layer on the cell surface (Nimi et al., 1983). Other gram-positive organisms such as Mycobacterium, Brevibacterium, Streptomyces and Streptococcus are also resistant to treatment with lysozyme. In the case of Mycobacterium and Streptomyces, protoplasts have been obtained by first treating the cells with glycine in growth medium (Baltz, 1978; Hopwood, 1981; Udou et al., 1982). The glycine is believed to replace alanine in the peptidoglycan, thereby interfering with crosslinking. Cells treated in this manner become more sensitive to lysozyme. Lysozyme and  $\alpha$  amylase were used together to obtain protoplasts of Streptococcus

lactis (Okamoto et al., 1983). Protoplasts have been obtained in lysozyme-resistant Brevibacterium and Corynebacterium by treating with penicillin in combination with lysozyme either in a sequential manner or simultaneously (Kaneko and Sakeguchi, 1979; Shtannikov et al., 1981; Rytir et al., 1982). Most gram-negative bacteria peptidoglycan is sensitive to lysozyme (Schaitman, 1981). However, the peptidoglycan is surrounded by a lysozyme-impermeable outer membrane, which must first be destroyed before the lysozyme can act. Digestion of the peptidoglycan does not result in the removal of this outer membrane. Therefore, such osmotically-sensitive structures are more appropriately termed spheroplasts. Removal of the outer membrane to produce true protoplasts is an important and probably essential step if fusion is to occur. In gram-negative bacteria such as E. coli, a procedure has been developed involving lysozyme treatment and EDTA that will produce true protoplasts (Weiss, 1976). Spheroplasts with about 15% of exposed cytoplasmic membrane of Providencia alcalifaciens were produced using a combined glycine and lysozyme EDTA treatment (Cortzec et al., 1979). These were successfully utilized in subsequent fusion experiments.

Conditions for regeneration of protoplasts appear to be far more complex than for their development and may vary even in closely related species (Hopwood, 1981). Results of Fodor and Alföldi (1979) emphasize that various

physiological factors can greatly affect regeneration rates. They claim complete distortion of genetic information derived from their fused phenotypes due to variable regeneration of the parental mutant types. Gabor and Hotchkiss (1982) also noted physiological factors such as crowding on plates played an important role in regeneration of fused protoplasts, thus supporting observations of Fodor and Alfoldi. In an earlier study by Landman et al. (1968) crowding was found to delay reversion markedly. In studies with Streptomyces by Baltz and Mutsushima (1981), both the temperature of cell growth prior to protoplast development and the temperature during regeneration were found to effect regeneration rates. The optimum growth temperatures required for good regeneration varied from species to species and was not necessarily the same as that temperature optimum required during regeneration. In addition dehydration of regeneration plates and plating by using a soft agar overlay was said to result in a more rapid, synchronous and efficient regeneration. As a result auto-inhibition exhibited by some species was overcome. Using optimal conditions for strains tested nearly 100% regeneration was achieved.

Careful examination of parameters for the generation of Bacillus subtilis have resulted in improvements to the established hypertonic media that can obtain up to 100% regeneration rates (Gabor and Hotchkiss, 1979). The primary factor responsible for this high rate was the

addition of 1% bovine serum albumin (BSA) to the growth and dilution media. Unfortunately regeneration did drop to 10 to 75% after treatment with PEG. BSA was also reported to enhance regeneration of Staphylococci. However, the use of BSA in the regeneration media precludes the direct selection of recombinants. Akamatsu and Sekiguchi (1984) developed a method of regeneration of Bacillus species protoplasts using a preincubation with 3% polyvinylpyrrolidone. No appreciable growth of auxotrophs was obtained whereas regeneration frequencies increased 500-fold in some cases. In addition, a 20 fold increase in regeneration rates was obtained when an agar overlay method was used as opposed to spread plates. Lower agar concentrations also resulted in increased regeneration frequencies.

Another interesting observation made by Akamatsu and Sekiguchi (1984) was that regenerating protoplasts increased in size first then underwent a non-oriented division. In regenerant colonies which had been plated using an overlay method, cells were in the bacillary form in 3 or 4 days. When the protoplasts were plated using a spread plate method the majority stopped growing after a few divisions. Akamatsu and Sekiguchi (1984) hypothesized that the newly synthesized cell wall components at high concentrations around the protoplasts might be an important factor for their regeneration.

Past studies also indicate a possible role of cell wall components in the regeneration of protoplasts. Differences in the envelope structure of three different L-forms of Proteus mirabilis were examined by Demonty et al. (1973). An incomplete cell wall about 8 nm thick outside the plasma membrane was observed in two unstable L-forms whereas the stable L-form was enveloped only by the plasma membrane. The remaining cell wall of the unstable L-forms was believed to be made up largely of lipopolysaccharides. Peptidoglycan content did not correlate to the instability of the various L-forms.

Reversion of Bacillus subtilis was stimulated by the presence of cell wall extractions and a variety of autoclaved intact microorganisms (Landman and Forman, 1969).  $Mg^{2+}$  and  $K^{+}$  ions were required for reversion. Protoplasts were said to enlarge but not divide in liquid medium and reversion was stimulated with gelatin or hard agar. Reversion was studied via the use of various antibiotics blocking DNA, RNA, protein, and cell wall synthesis in order to determine if and when these events were required. Reversion was broken down into three steps. It was speculated that the first step involved an alteration of the membrane, the second step, the depression of DAP and mucopeptide wall synthesis, and the last step, teichoic acid synthesis. The physical immobilization of excreted cell products at the protoplast surface early in

step two was also believed to be important.

Conditions for regeneration of Brevibacterium have been examined. Best reversion rates when succinate is used as to osmotic stabilizer (as opposed to sucrose) and when the protoplasts are imbedded in the top of a semisoft 0.8% agar layer (Shtannikov et al., 1981). Enrichment with casein hydrolysate or amino acids and nitrogen bases also increased reversion with reported reversion rates being 20 to 70%. The results correspond fairly well to those in an earlier work by Kaneko and Sakaguchi (1979) where a 30% regeneration rate was reported under similar conditions of regeneration. No other variables that might affect regeneration of Brevibacterium protoplasts were investigated.

As mentioned earlier, fusion of protoplasts have been accomplished with both procaryotes and eucaryotes. Fusion has not been limited to fusion of protoplasts of the same species, but can be made to occur with different species and genus (Cocking et al., 1981; Ferenzy, 1981; Hales, 1977; Hopwood, 1981; Shepard et al., 1983). Interspecific protoplast fusion has been reported more often for eucaryotes than for procaryotes. Presumably, this does not stem from the inability to achieve interspecific protoplast fusion but rather simply that few have tried it. Hopwood (1981) reports attempts at interspecific fusion with Streptomyces and concludes that although such recombinants

occur, they occur at very low frequency and are not very stable. Shtannikov et al. (1981) report that fusion does occur between Corynebacterium glutamicum and Brevibacterium flavum. However, it should be noted that these two genus are so closely related that some researchers indicate they are in fact the same. Interspecific fusion has also been reported for Bacillus species (Akamatsu and Sekiguchi, 1983). Numerous examples of intraspecies fusion occur in the literature and have been reviewed in some detail (Ferenezy, 1981; Hopwood, 1981; Pebery, 1980).

Fusion itself is thought to occur in distinct phases (Ahkong et al., 1975; Frehel et al., 1979; Gumpert, 1980; Knutton and Pasternak, 1979). The model proposed by Gumpert (1980), which also encompassed those proposed by others, specifies five steps. The first is the formation of a contact zone. Presumably, this is promoted by fusogens such as polyethylene glycol (PEG). PEG is said to promote aggregation of the protoplasts (Tilcock and Fisher, 1982). The second step is the establishment of molecular contacts and alteration of membrane structures. Changes in the bilayer phase of membrane lipids have been reported by Cullis and Hope (1978 and 1981) using  $^{31}\text{P}$ -NMR techniques. This alteration of the membrane includes increased fluidity of the phospholipids and lateral diffusion of intrinsic proteins which may be promoted by the action of both PEG and  $\text{Ca}^{2+}$  (Tilcock and Fisher, 1979). PEG has been shown to

cause changes in phospholipid hydration and polarity which may account for these alterations (Arnold et al., 1983). In particular, it may be the removal of water associated with the bilayer that is the principle destabilizing effect which results in fusion (Gibson and Strauss, 1984). The third step is the formation of a fusion membrane and the fourth the formation of a separation layer by membrane material. The final step is the migration of lipid vesicles and intermixing of cell contents. Success of fusion may depend in part on the partial removal of the PEG (Ferenezy, 1981; Wojcieszyn et al., 1983).

Some controversy does exist on the ability of PEG by itself to cause fusion. Honda et al. (1981a) reported that purified PEG mw 6000 was unable to cause cell fusion. They concluded that the commercial grade PEG contained at least two components, one which caused aggregation and one which resulted in the perturbation of the phospholipid bilayer. Honda et al. (1981b) later identified the components removed during PEG purification as antioxidants like  $\alpha$ -tocopherol or other phenolic derivatives which are often added to commercial PEG. Fusion activity could be restored to the purified PEG when such components were added. These results were supported by the work of Wojcieszyn et al. (1983). In contrast, Smith et al. (1982) reported that four out of five commercial preparations of PEG retained fusogenic activity

upon purification. Although the addition of small quantities of compounds of the type utilized by Honda et al. (1981b) was observed to enhance cell fusion up to 50%. PEG is now used almost exclusively to facilitate fusion. Factors investigated included pH, concentration of  $\text{Ca}^{2+}$ , molecular weight of the PEG, time and temperature of treatment. Generally speaking, more concentrated solutions of 50% PEG (wt/vol) are more effective than dilute solutions. For fusion of mammalian cells, PEG mw 6000 have been used (Pontecorvo, 1975). In later procedures, DMSO was added to enhance fusion by 41.7% (wt/vol) PEG at mw 1000 (Hales, 1977).

For fusion of various Streptomyces, PEG from 1000 to 6000 has been used with and without the addition of DMSO. PEG was generally in the range of 36 to 50% (Akamatsu and Sekiguchi, 1983; Hopwood and Wright, 1978; Hopwood and Wright, 1981; Ochi et al., 1979; Rose, 1981). In fusion experiments with Bacillus species, PEG 6000 is most often used (Fodor and Alfoldi, 1976; Fodor and Alfoldi, 1979; Frehel et al., 1979; Horvath, 1971; Schaeffer et al., 1976). With Brevibacterium, PEG 6000 was also used at 33% by Kaneko and Sakaguchi (1979) and at 40% by Shtannikov et al. (1981). Fusion by Shtannikov et al. (1981) was done at room temperature, whereas Kaneko and Sakaguchi (1979) used 30°C. The possible effect of different temperatures was not investigated by either group. Kaneko and Sakaguchi (1981) report that pH did not much effect the fusion

mixture. The range that they tested is not given. Shtannikov et al. (1981) did not vary pH conditions. Kaneko and Sakaguchi (1979) did test exposure time to PEG and report no effect on fusion frequency over a 1 to 5 minute period. Neither group investigated the possible influence of  $\text{Ca}^{2+}$  on the fusion of Brevibacterium. Controls utilizing DNase to assure that genetic transfer was indeed due to protoplast fusion as opposed to DNA uptake are not mentioned. A requirement for  $\text{Ca}^{2+}$  has been noted by groups working with fungi, but it has not been well investigated in fusion with bacteria (Ferenezy, 1981; Peberdy, 1980).  $\text{Ca}^{2+}$  has been shown to be involved with the fusion of artificial membranes in a number of studies (Ingolia and Koshland, 1978; Ito and Ohnishi, 1974; Papahadiopoulos et al., 1974; Papahadiopoulos et al., 1976; Sun et al., 1979). The mechanism of action of the  $\text{Ca}^{2+}$  is thought to be by causing phase separation or disturbances in the lipid molecules. This could result in the formation of small bridges when cells come into contact, which then enlarge for fusion.

It should be pointed out at this time that fusion does not by itself ensure genetic exchange. Recombination is a separate event. Frehel et al. (1979) placed emphasis on an optimal post-PEG incubation period. The method of selection of recombinants may exert a strong influence on recombinant genotypes recovered. Generally, both direct and indirect

screening methods have been used. Fodor and Alföldi (1979) used a direct method, screening on the basis of nutritional requirements. As mentioned previously, this led to considerable bias in the recovery of the recombinants. Under the circumstances, an indirect method might produce better results.

With Brevibacterium, direct selection for antibiotic markers has been utilized (Kaneko and Sakaguchi, 1979; Shtannikou et al., 1981). Recombinants are selected on the basis of rifampsin and streptomycin-resistance markers obtained from each parental type, then classified on the basis of a non-selected auxotrophic marker. Presumably, this could avoid physiological bias introduced by variable nutrient content in the regeneration media as utilized by Fodor and Alföldi (1979).

In the literature, there are a few notable novel approaches to selection of recombinants. These involve the selection of one or both the parental protoplasts so that only the resulting recombinant progeny will grow. One method by Hopwood and Wright (1981) involves the use of ultraviolet irradiated protoplasts. The ultraviolet radiation presumably leaves the protoplast intact but nonviable unless fused with another protoplast not carrying the same defect. This is the same reasoning used by Wright (1978). Here cells were given lethal doses of a specific reagent or inhibitor, which selectively damages a specific

molecule. The two parental types are then fused and only those recombinants receiving a full set of undamaged molecules can survive, thus eliminating the need for selectable markers.

The fusion of nonviable parental organisms gives rise to another method of genetic transfer involving the fusion of protoplasts with phospholipid vesicles containing genetic information. Such a method has been developed by Fraley et al. (1979). In this study liposomes containing pBR322 DNA were fused with E. coli. Such a procedure could be adapted for use with other microorganisms.

In addition to fusion of whole protoplasts and of liposomes with protoplasts, a great deal of success has been achieved with the transformation of protoplasts with DNA (Akamatsu and Sekiguchi, 1982; McDonald and Burke, 1984; Kondo and McKay, 1982). The procedures used are generally similar to those used for protoplast fusions.

Protoplast fusion and transformation have become increasingly important as a means to improve strains of organisms for which no other genetic exchange system exists. There are presently numerous examples in the literature where these methods are now being applied to improve or enhance various properties of organisms with no other exchange system. A second purpose for the development of such systems is to develop a functional map of the bacterial chromosome. One notable effort has been made in this

direction by Stahl and Pattee (1983a, 1983b). Location of various markers was first done using protoplast fusion. Calculation of frequencies of recombinants and possible linkages of markers was done directly by entering results of 9 and 10 factor crosses into a computer. Results were then confirmed by transformation of competent cells of Staphylococcus aureus.

In summary, protoplast fusion has been utilized for the exchange of genes and the mapping of the chromosome in a variety of organisms. Because other avenues of genetic exchange do not appear to exist for Brevibacterium lactofermentum, protoplast fusion could fill this void. In addition, protoplast fusion can provide a method of mapping for Brevibacterium lactofermentum. As such protoplast fusion could provide a convenient method for strain improvement via the genetic manipulation of this microorganism.

## MATERIALS AND METHODS

### Strains

Brevibacterium lactofermentum strain 2256 was kindly supplied by Dr. H. Momose, Central Research Laboratories, Ajinomoto Co., Japan. Mutants derived from this strain were obtained by N-methyl-N-nitro-N-nitrosoguanidine (NTG) mutagenesis. Str<sup>R</sup> strains were resistant to 100 µg/ml streptomycin. Rif<sup>R</sup> strains were resistant to 10 µg/ml rifampsin.

### Media

Complex media (CM) was that used by Momose and Takagi (1978) and had the following composition per liter: yeast extract, 10.0 g; glucose, 5.0 g; NaCl, 5.0 g; and polypeptone, 10.0 g at pH 7.0. Minimal media (MM) per liter composition was: glucose, 20.0 g; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 10.0 g; urea, 2.5 g; KH<sub>2</sub>PO<sub>4</sub>, 1.0 g; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.4 g; biotin, 50.0 µg; thiamin-HCl, 200 µg; NaCl, 50.0 mg; FeSO<sub>4</sub>·7H<sub>2</sub>O, 10.0 mg; at pH 7.0. Plates of CM or MM media contained 2% agar. Amino acids were added to a final concentration of 0.01% as required by the particular autotrophic strain. Selective plates for selection of recombinants were CM with 100 µg/ml

streptomycin and 10  $\mu$ g/ml rifampsin or MM with 100  $\mu$ g/ml streptomycin and 10  $\mu$ g/ml rifampsin and appropriate amino acid(s) at a final concentration of 0.01%. Buffer for the dilution of protoplasts was a modification of sucrose-maleate (SMM) buffer used by Wyrick and Rogers (1973). It contained 0.41 M sucrose, 20 mM maleate and 0.2M  $\text{MgSO}_4$  at pH 6.5. All other dilutions were carried out using 0.1 M potassium phosphate buffer at pH 7.0. Lysozyme treatment solutions were either MM with 10 mg/ml lysozyme, 0.2  $\mu$ /ml penicillin and 0.41 M sucrose or a modification of that used by Chang and Cohen (1979) which consisted of SMM plus 35 g/l Difco pennassay media (SMMP) with 10 mg/ml lysozyme, 0.3  $\mu$ /ml penicillin, and 0.41 M sucrose. Filter sterilized BSA, 0.1% (w/v) final concentration was added to stabilize protoplasts. Lysozyme solutions were made fresh prior to each use and filter sterilized. Two types of regeneration media were used. Minimal regeneration media was MM with 135 g/l Na succinate. Complex regeneration media (CR) was a modification of that utilized by Wyrick and Rogers (1973) with a per liter composition as follows: Tris, 12.0 g; KCl, 0.5 g; glucose, 10.0 g;  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 8.1 g;  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 2.2 g; (group A),  $\text{Na}_2$  succinate, 135 g; (group B), peptone, 4.0 g; yeast extract 4.0 g; casamino acid, 1.0 g;  $\text{K}_2\text{PO}_4$ , 0.2 g; (group C). Agar, 0.45% final concentration, was added to group A. Each group was brought to pH 7.0 and autoclaved separately, group A and B at  $115^\circ\text{C}$  and group C

at 120°C. Once again, BSA at 0.1% final concentration was added to stabilize protoplasts.

#### Protoplast Formation

Development of protoplast procedure was modified from those utilized by Kaneko and Sakaguchi (1979) and Shtannikov et al. (1981). An overnight culture grown in either MM or CM was inoculated into the same type media to an initial OD<sub>(575)</sub> of 0.150 - 0.200 and incubated on a shaker at 32°C until an OD<sub>(575)</sub> of approximately 0.600 was reached. This OD corresponds to about 10<sup>8</sup> cells/ml. Fresh filter sterilized penicillin was added to give a final concentration of 0.3 u/ml. Cells were cultivated for one more generation period (1.5 - 2.0 hrs), then washed one time with SMM buffer, resuspended in one of the lysozyme solutions and incubated without shaking at 32°C overnight. Rate of protoplast development was determined by taking samples from lysozyme solution at various intervals and diluting with potassium phosphate buffer to lyse all osmotically sensitive cells. Samples were then plated on CM to determine the number of osmotically insensitive cells remaining.

#### Reversion of Protoplasts

To revert protoplasts, lysozyme treated cells were washed one time with SMM plus BSA and resuspended in a volume of SMM equal to the starting volume. The sample was then diluted appropriately in SMM plus BSA and plated in 10 mls

of regeneration media plus BSA. Plates were incubated upright at 32°C for up to 2 weeks to ensure all protoplasts capable of reversion had grown. This number represented regenerated protoplasts plus any remaining osmotically insensitive cells. To obtain % regeneration, this total was corrected for the number of cells remaining osmotically insensitive as was the initial number prior to lysozyme treatment.

$$\% \text{ regeneration} = \left( \frac{a-b}{c-b} \right) \times 100$$

where

a = population from regeneration plates

b = population from "whole cell" plates

c = initial population

The number of reverted protoplasts was then divided by the corrected initial number, and multiplied by 100 to give the % regeneration.

#### Microscopic Observation of Protoplasts

Protoplast development was monitored on a phase microscope. Direct cell and protoplast counts were done with a Petrof-Hauser counting chamber. Protoplast reversion was observed by taking samples at various time intervals from the soft agar regeneration media plates, placing on a slide and covering with a cover slip.

#### Protoplast Fusion

Protoplasts of two strains, Met<sup>-</sup>rif<sup>R</sup> and Arg<sup>-</sup>str<sup>R</sup>, or Thr<sup>-</sup>rif<sup>R</sup> and Arg<sup>-</sup>str<sup>R</sup> were mixed (1 ml each type) and

centrifuged. They were then resuspended in one-tenth the volume of SMMP at 37°C. Two mls of polyethylene glycol (PEG mw 6000, MCB, 33% w/v in SMM without MgCl) and filter sterilized) prewarmed to 37°C was added and rapidly mixed with the protoplasts. The solution was then incubated 30 mins at 37°C. After incubation, 8 mls of SMMP was added to the PEG - protoplast suspension. Protoplasts were then centrifuged and resuspended in 2 mls of SMMP. The fused protoplasts were then plated for nonselective regeneration by plating 1 ml per plate of 10 mls regeneration media. These plates were incubated 3 days at 32°C. After 3 days, 10 mls phosphate buffer (0.1 M, pH 7.0) was added to each plate and homogenized with the agar by quickly drawing the suspension up and down a 10 ml pipet. Once homogenized, the sample was diluted appropriately and spread plated on CM plates to determine the population of regenerated cells and on selective media to determine fusion and recombination. Controls were run in exactly the same manner except 2 mls of only one type mutant was used per fusion. To determine if protoplasts were transformed as well as fused, DNase I (Sigma) was added to the protoplast mixture at a final concentration of 10 mg/ml prior to and during PEG treatment in some experiments.

#### Radiolabeling of Cell Wall

Cells were grown overnight in MM plus 0.01% lysine and transferred to fresh MM plus 0.01% lysine plus 100 µl of

of  $^3\text{H}$ -DL-mesodiaminopimelic acid (Research Products International Corp., Mt. Prospect, Illinois,  $2\ \mu\text{Ci}/\mu\text{l}$   $32\ \mu\text{Ci}/\text{mMol}$ ) at a starting  $\text{OD}_{(575)}$  of .2. Cells were then cultivated and protoplasted as before. One ml samples were taken prior to lysozyme treatment and at various time intervals during lysozyme treatment. Controls were treated in exactly the same manner except lysozyme was left out of the lysozyme solution. Samples were centrifuged and washed 2 times with SMM and resuspended in 1 N HCl. 0.5 mls of each sample (duplicates) were placed in lypholization vials, flushed with  $\text{N}_2$ , and sealed. The sealed vials were heated at  $110^\circ\text{C}$  overnight. Ten  $\mu\text{l}$  of each hydrolysed sample was counted directly in a 20 ml glass vial with 15 mls of aqueous scintillation cocktail (RPI Safety solve). Fifty  $\mu\text{l}$  of each hydrolysed sample was chromatogramed on thin layer cellulose plates. The solvent system used was methanol:  $\text{H}_2\text{O}$ :pyridine:12NHCl (100:35:20:5). Each chromatogram was cut into 0.5 x 1.0 inch fractions. The cellulose was scraped off each fraction and placed into a 20 ml glass vial with 15 mls of nonaqueous scintillation cocktail (RPI 3a20). These samples were left overnight in the dark at room temperature prior to counting. Amino acid standards were run with the same solvent system and sprayed with 1% ninhydrin in acetone and heated briefly to visualize their locations. DAP was identified by its characteristic green spot.

## RESULTS

### Protoplast Formation

Logarithmically growing wild type cells of B. lactofermentum were subjected to variable concentrations of penicillin pretreatment and lysozyme-penicillin treatment in order to determine optimal conditions for the development of protoplasts (Table 1). When treated with lysozyme only, osmotic sensitivity increased with increasing amounts of lysozyme. However, microscopic examination indicated poor (<25%) protoplast development at all concentrations of lysozyme used. When penicillin was added to the procedure, the proportion of protoplasts increased with increasing amount of penicillin used. Penicillin at concentrations  $\leq 0.3$  units/ $10^8$  cells did not inhibit growth. Attempts to replace penicillin treatment with 2% glycine gave the same results as treatment with lysozyme only, regardless of the length of preincubation in media with 2% glycine. Attempts to use stationary phase cells grown overnight in penicillin resulted in greater than 99% of the population sensitive to osmotic shock but only approximately 50% appeared to be free protoplasts under microscopic observation.

Utilizing 0.3 units/ml penicillin and 10 mg/ml lysozyme at a starting population of  $10^8$  cells/ml, protoplast

Table 1. Development of osmotic sensitivity in Brevibacterium lactofermentum and appearance of protoplasts.<sup>a</sup>

| Concentration<br>g lysozyme<br>(mg/ml) | Concentration of penicillin<br>(units/ml) |                                                  |                                                 |                                                  |                                                 |                                                  |
|----------------------------------------|-------------------------------------------|--------------------------------------------------|-------------------------------------------------|--------------------------------------------------|-------------------------------------------------|--------------------------------------------------|
|                                        | 0                                         |                                                  | 0.1                                             |                                                  | 0.2                                             |                                                  |
|                                        | % whole<br>cells<br>remain-<br>ing        | proto-<br>plast<br>develop-<br>ment <sup>c</sup> | % whole <sup>b</sup><br>cells<br>remain-<br>ing | proto-<br>plast<br>develop-<br>ment <sup>c</sup> | % whole <sup>b</sup><br>cells<br>remain-<br>ing | proto-<br>plast<br>develop-<br>ment <sup>c</sup> |
| 1                                      | 38                                        | +                                                | 2.1                                             | +                                                | 0.67                                            | 0.52 ++                                          |
| 5                                      | 0.80                                      | +                                                | 0.029                                           | ++                                               | 0.014                                           | 0.011 +++                                        |
| 10                                     | 0.013                                     | +                                                | 0.010                                           | ++                                               | 0.010                                           | 0.010 +++                                        |

<sup>a</sup>Cells were grown in MM and treated with lysozyme in SMMP for 20 hours. Penicillin treatment was begun 2 hours prior to lysozyme treatment and continued throughout lysozyme treatment. Cell population at the start of lysozyme treatment was about 10<sup>8</sup>/ml.

<sup>b</sup>Whole cells remaining was determined by phosphate buffer and plating on CM.

<sup>c</sup>Protoplast development was determined by microscopic observation. Key: + = <25%, ++ = 25-75%, +++ = >75%.

development appeared nearly complete in 3 - 4 hours (Figure 1). Osmotic sensitivity showed greatest increases in the first few hours and at 4 hours only approximately 0.1% of the starting population remained osmotically insensitive (Figure 2). A decrease in  $OD_{(575)}$  paralleled development of protoplasts (Figure 2 inset).

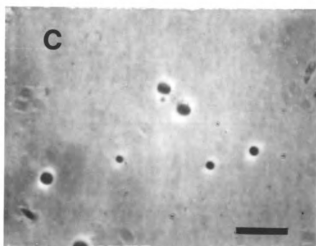
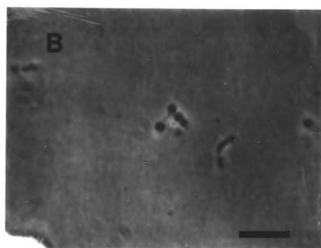
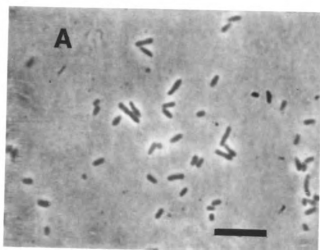
The effects of growth media (CM vs MM) and lysozyme treatment medium on the protoplast development were examined (Table 2). MM resulted in substantially fewer cells (10 times) remaining osmotically insensitive than did CM. However, the media used during lysozyme treatment did not appear to have a marked effect on protoplast development.

Sucrose concentration has been reported to have an effect on protoplast development (Kaneko and Sakaguchi, 1979). Two different concentrations were used in this study to determine the optimal one (Table 3). No difference in the development of protoplasts was detected regardless of the media used to cultivate the cells. However, a slight advantage was detected in the regeneration when 0.41 M sucrose was used as opposed to .5 M sucrose.

#### Regeneration of Protoplasts

Protoplasts were plated in CR and examined microscopically after 48 hours of incubation. Two types of patterns were detected (Figure 3). The first was a large swollen protoplast-like form with few protrusions that appeared to be regenerated cells. The second was smaller more numerous

Figure 1. Development of protoplasts. A. Normal cells of Brevibacterium lactofermentum; B. After 3 hours in lysozyme solution; C. After 16 hours in lysozyme solution. The bar represents 10  $\mu\text{m}$ .



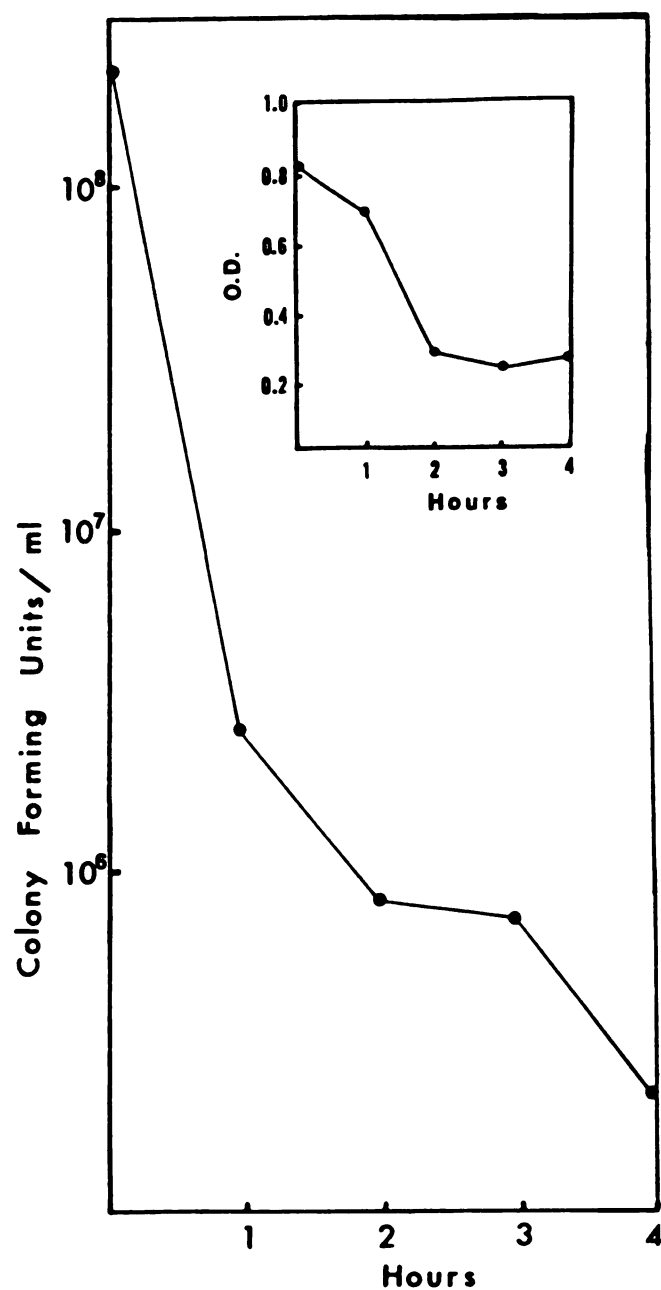


Figure 2. Appearance of osmotic sensitivity during lysozyme treatment. Population of whole cells remaining was determined by dilution in phosphate buffer and plating on CM. Inset: change in OD(570) during lysozyme treatment.

Table 2. Effect of media on the development and regeneration of protoplasts<sup>a</sup>

| Growth Media | Lysozyme Treatment Media | Regeneration Media | % Whole Cells Remaining | % Regeneration |
|--------------|--------------------------|--------------------|-------------------------|----------------|
| CM           | SMP                      | CR                 | $1.5 \times 10^{-1}$    | 1.2            |
| CM           | SMP                      | MM                 | $1.5 \times 10^{-1}$    | 0.61           |
| CM           | MM                       | CR                 | $3.3 \times 10^{-1}$    | 0.61           |
| CM           | MM                       | MM                 | $3.3 \times 10^{-1}$    | 0              |
| MM           | SMP                      | CR                 | $5.2 \times 10^{-2}$    | 12             |
| MM           | SMP                      | MM                 | $5.2 \times 10^{-2}$    | 13             |
| MM           | MM                       | CR                 | $4.0 \times 10^{-2}$    | 7.5            |
| MM           | MM                       | MM                 | $4.0 \times 10^{-2}$    | 9.1            |

<sup>a</sup> Cells grown and penicillin treated in growth media indicated. Lysozyme treatment and regeneration were in media indicated. Abbreviations as indicated previously. % whole cells remaining and % regeneration determined as before.

Table 3. Effect of sucrose concentration on protoplast development and regeneration.

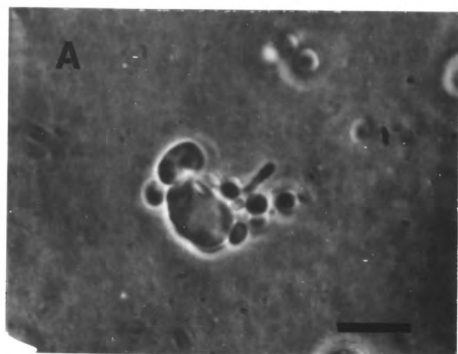
| Media                     | % whole cells <sup>c</sup><br>remaining | % regeneration <sup>c</sup> |
|---------------------------|-----------------------------------------|-----------------------------|
| Experiment 1 <sup>a</sup> |                                         |                             |
| .5 M sucrose              | $3.0 \times 10^{-2}$                    | 0.4                         |
| .41 M sucrose             | $4.0 \times 10^{-2}$                    | 1.5                         |
| Experiment 2 <sup>a</sup> |                                         |                             |
| .5 M sucrose              | $1.6 \times 10^{-2}$                    | 1.1                         |
| .41 M sucrose             | $5.9 \times 10^{-3}$                    | 1.2                         |
| Experiment 3 <sup>b</sup> |                                         |                             |
| .5 M sucrose              | $6.3 \times 10^{-3}$                    | 3.2                         |
| .41 M sucrose             | $9.4 \times 10^{-3}$                    | 5.7                         |

<sup>a</sup> Cells were grown and penicillin treated in CM and transferred to lysozyme in SMP for development of protoplasts.

<sup>b</sup> Cells were grown and penicillin treated in MM and transferred to lysozyme in SMP for development of protoplasts.

<sup>c</sup> Whole cells remaining was determined by dilution in phosphate buffer and plating on CM. % regeneration was determined by dilution in SM and plating on CR.

Figure 3. Regeneration process of protoplasts. Protoplasts were plated in CR. After 48 hours of incubation (32°C) samples were withdrawn and inspected.  
(A) Large type protoplast that often developed.  
(B) Small protoplast with larger amounts of regeneration. The bar represents 10  $\mu\text{m}$ .



protoplast-like forms with many pleomorphic cells in close proximity in addition to what appeared to be regenerated cells. Colonies on CR plates usually appeared in 3 to 5 days. However, plates were kept incubated two weeks to ensure that any reverting protoplasts had sufficient time to develop. Microscopic examination of colonies on CR plates revealed a variety of pleomorphic forms in addition to normal rods and some remaining coccoid shapes.

Agar depth appeared to effect regeneration rates of the protoplasts (Figure 4). Maximal regeneration rates appeared to be at 10 mls of agar per petri dish. Rates dropped sharply with increasing agar depth after 10 mls. The addition of catalase (.02% final concentration) or penicillinase (1 unit/ml final concentration) to CR had no effect on regeneration.

Effect of growth media on the development of protoplasts was previously pointed out as quite dramatic (Table 3). There was also a noticable effect of this parameter on regeneration. Regeneration rates ranged from 13-7.5% when MM was used as the growth media as opposed to 1.2-0% when CM was used as growth media. In addition, although lysozyme treatment, as stated previously, did not appear to effect protoplast development, it did appear to affect regeneration. Regeneration rates were approximately 2 fold higher when SMMP was used for lysozyme treatment than when MM was used.

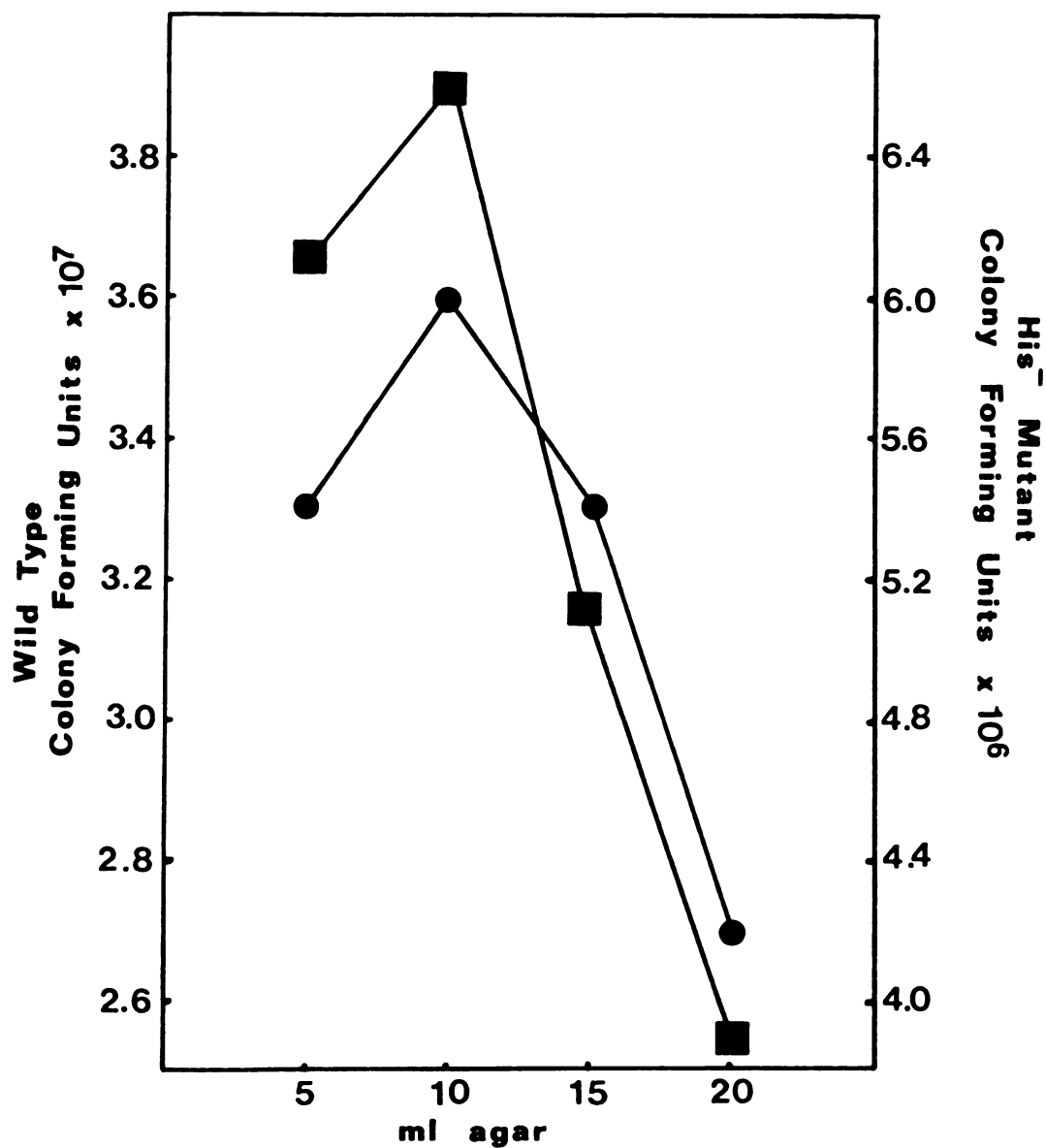


Figure 4. Regeneration of wild type and His-mutant protoplasts vs depth of agar: Protoplasts were pour plated with various agar amounts into petri plates. Wild type  $\bullet$ — $\bullet$ , His-mutant  $\blacksquare$ — $\blacksquare$ .

Regeneration media (CR vs MM) itself had a slight effect on regeneration rates. CR appeared to have a slight advantage when CM was used as the growth media. When MM was used as the growth media the tendency was reversed.

Finally, the effect of bovine serum albumin (BSA) on the regeneration of protoplasts was tested (Table 4). In each of three separate experiments where BSA was added at a final concentration of 0.1% w/v to both the lysozyme treatment and regeneration media, approximately a 3 fold increase in regeneration rates was observed.

#### Variation in Mutants

All investigations of conditions for the development and regeneration of protoplasts were done using the wild type strain as a model. Optimized conditions were then applied to mutants developed for fusion. Figure 5 shows the rate of protoplast development measured as % osmotically insensitive cells remaining vs time for 3 different mutants plus the wild type. For each different mutant a different rate was observed. Regeneration rates of mutants appeared to be related to the completeness of protoplast development. This effect was further investigated by labeling studies with the wild type strain.

#### Determination of Remaining Cell Wall and Relationships to Regeneration

The relationship between fraction of cell wall remaining and regeneration was examined. Protoplasts were developed

Table 4. Effect of bovine serum albumin on the regeneration of protoplasts<sup>a</sup>

|              | % whole cell<br>remaining | % regeneration |
|--------------|---------------------------|----------------|
| Experiment 1 |                           |                |
| w BSA        | $7.2 \times 10^{-2}$      | 3.9            |
| w/o BSA      | $4.8 \times 10^{-2}$      | 1.2            |
| Experiment 2 |                           |                |
| w BSA        | $1.3 \times 10^{-1}$      | 1.2            |
| w/o BSA      | $3.0 \times 10^{-2}$      | .39            |
| Experiment 3 |                           |                |
| w BSA        | $2.1 \times 10^{-1}$      | 11             |
| w/o BSA      | $2.2 \times 10^{-1}$      | 3.4            |

<sup>a</sup>Cells were cultivated and penicillin treated in CM, lysozyme treated in SMP and regenerated on CR. BSA was added to lysozyme solution and CR at a final concentration of 0.1% w/v. % whole cells remaining and % regeneration was determined as before.

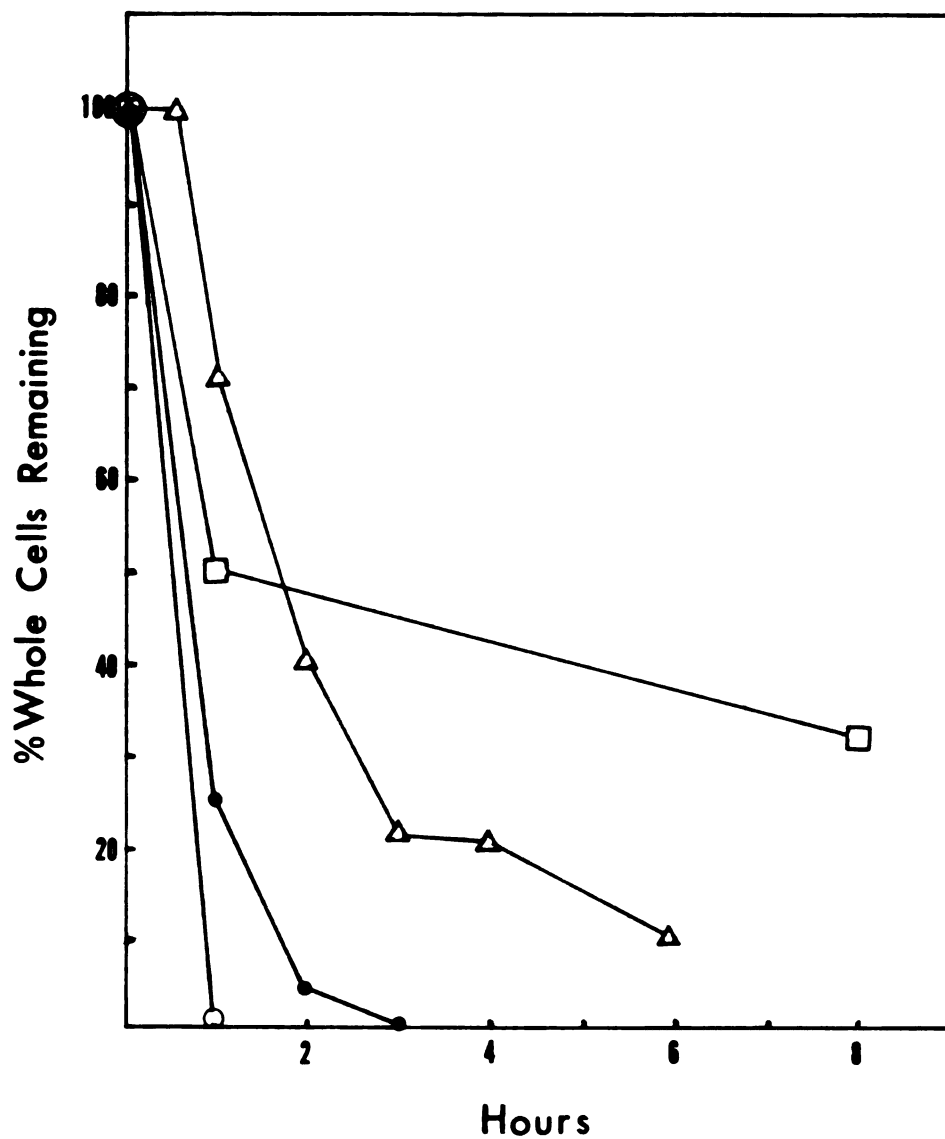


Figure 5. % Osmotically insensitive cells remaining vs length of lysozyme treatment. Cells were treated for the development of protoplasts as described in the materials and methods.

●—● Wild type. ▲—▲ Lys<sup>-</sup>. ○—○ His-Str<sup>R</sup>.  
 □—□ met<sup>-</sup>Rif<sup>R</sup>.

as stated in Materials and Methods. Concentrations of lysozyme and penicillin were varied in order to obtain intervals in which protoplast formation was still incomplete. Initial cell concentration was always  $10^8/\text{ml}$ .

In Figure 6 the results of 5 separate experiments with variable treatments as stated in the legend are plotted. It can be seen from these results that as total  $^3\text{H}$  decreases, the % regeneration also decreases. The correlation coefficient was 0.9 ( $P < .005$ ). Figure 7 illustrates the relationship between osmotic sensitivity and  $^3\text{H}$  remaining. Osmotic sensitivity appears to drop sharply after more than 40% of the  $^3\text{H}$  has been removed.

To determine that the total counts in samples were due to ( $^3\text{H}$ ) DAP, hydrolyzed samples from experiments using 5 mg/ml lysozyme with 0.2 units/ml penicillin were chromatographed and counted. To determine if any ( $^3\text{H}$ ) DAP was lost during the hydrolysis procedure and to determine location of ( $^3\text{H}$ ) DAP two controls were run as described in the legend of Figure 8. Both controls showed a single major peak at fractions 4-5 corresponding to an  $R_f$  of 0.24. DAP standard (unlabeled) were sprayed with ninhydrin and gave an  $R_f$  of 0.24. The  $R_f$  for lysine was found to be 0.45. The  $R_f$ 's of other amino acids were not determined since it has been previously reported that B. lactofermentum will only convert DAP to lysine and will not break down lysine (Tosaka and Takinami, 1978). In addition to the single major peak, a

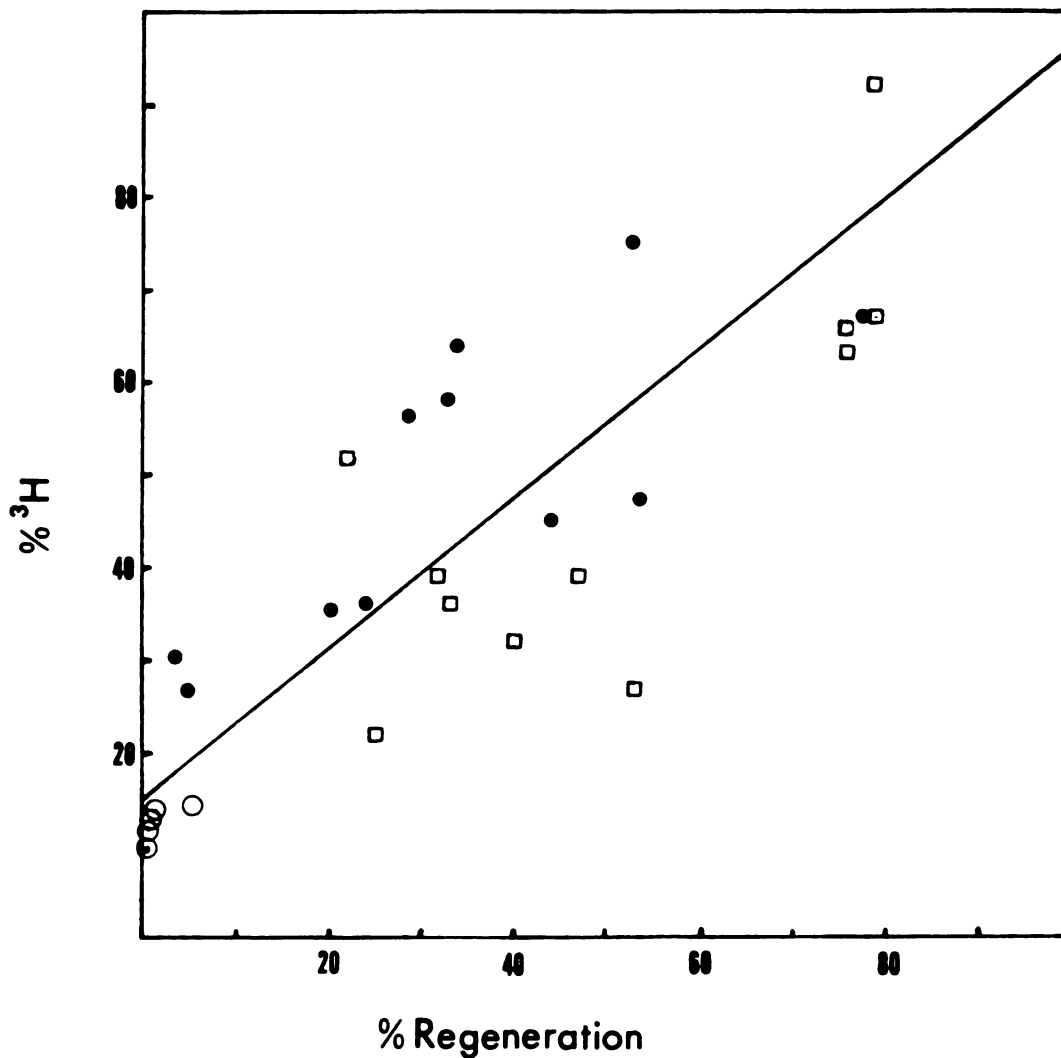
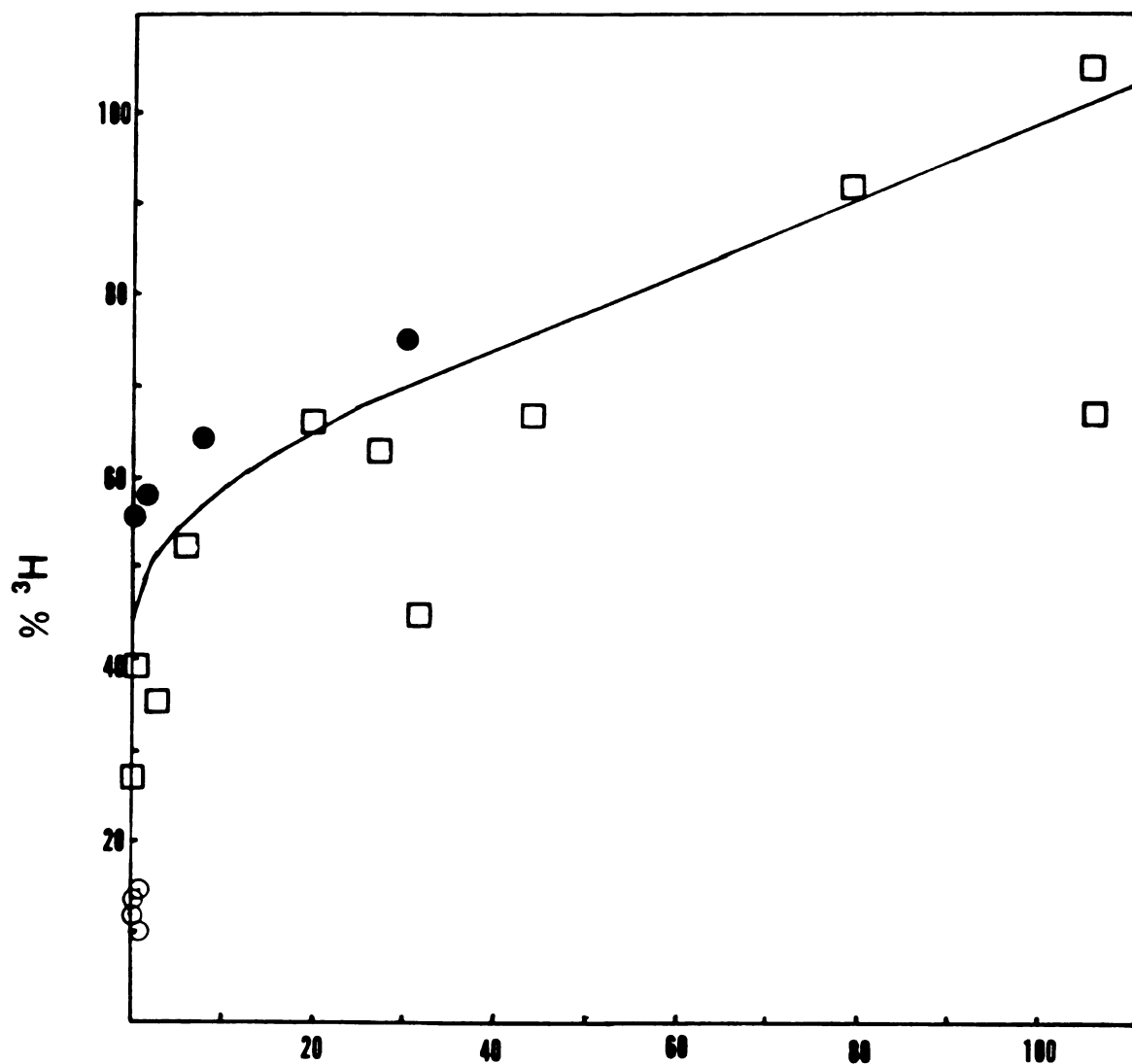


Figure 6. % <sup>3</sup>H in protoplasts vs % regeneration of protoplasts. Data was obtained from 5 separate experiments; 2 experiments where 1 mg/ml lysozyme and 0.2 units/ml penicillin was utilized, □; 2 experiments where 5 mg/ml lysozyme and 0.2 units/ml penicillin was utilized, ●; and 1 experiment where 10 mg/ml lysozyme and 0.3 units/ml penicillin was utilized, ○. Initial cell concentration was always 10<sup>8</sup>/ml. Procedure for development and regeneration of protoplasts as described in materials and methods. Slope of the line calculated to be 0.81 using linear regression. Calculated correlation was 0.9.



### % Whole Cells Remaining

Figure 7. %  $^3\text{H}$  in protoplasts vs % whole cells remaining. Data was obtained from 4 separate experiments. 1 experiment utilized 1 mg/ml lysozyme and 0.2 units/ml penicillin, ●; 2 experiments utilized 5 mg/ml lysozyme and 0.2 units/ml penicillin, □. 1 experiment utilized 10 mg/ml lysozyme and 0.3 units/ml penicillin, ○. Initial cell concentration was always  $10^8/\text{ml}$ . Procedure for development and regeneration of protoplasts as described in materials and methods.

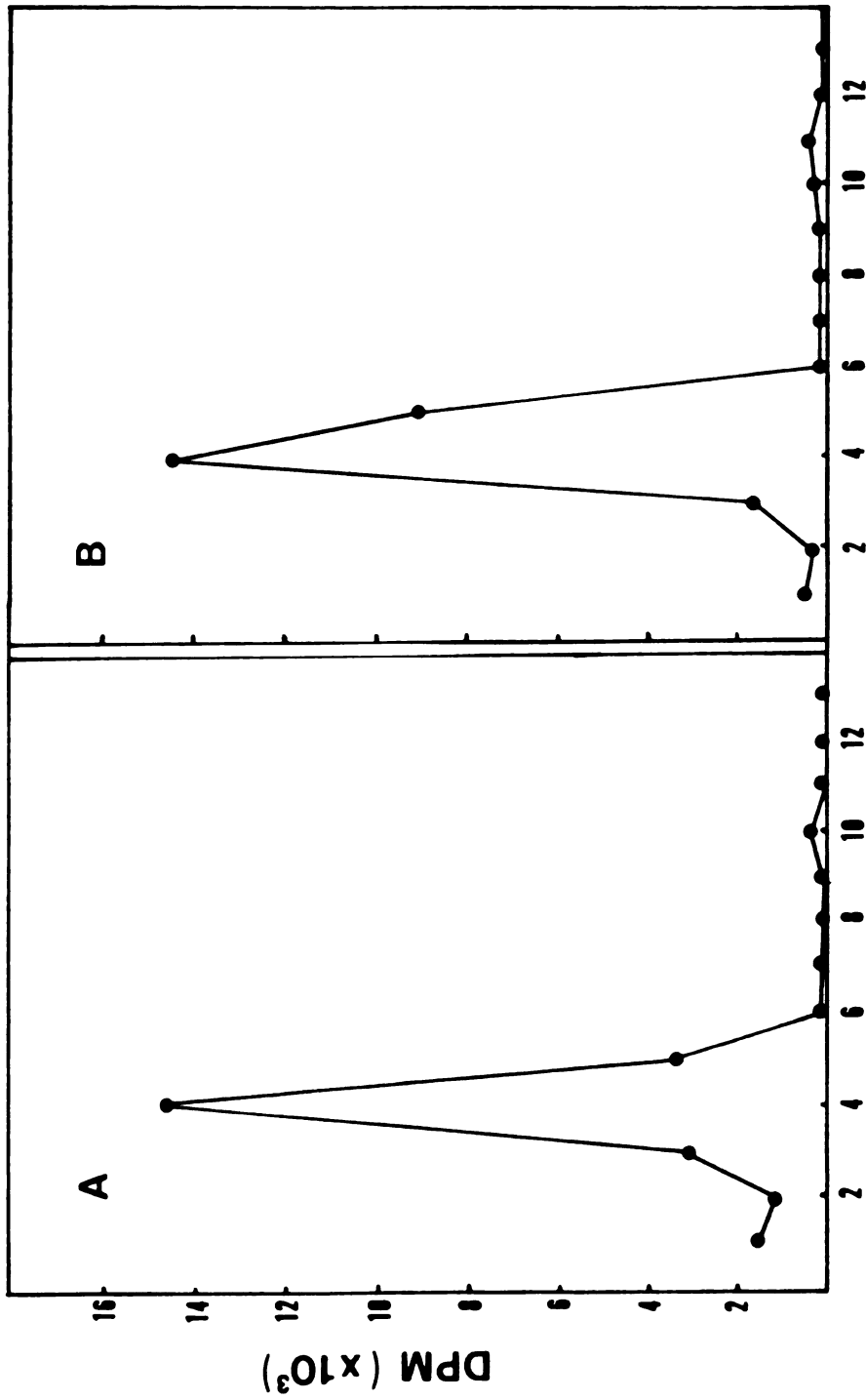


Figure 3A. Chromatogram of ( $^3\text{H}$ )DAP heated at  $110^\circ\text{C}$  overnight in 6 N HCl.

Figure 8B. Chromatogram of ( $^3\text{H}$ )DAP heated at  $110^\circ\text{C}$  with acid. Killed overnight in 6 N HCl.

minor contaminant was detected at fractions 10-11. This contaminant represented 1.3 and 1.7% of the total counts respectively.

When hydrolysed samples were chromatographed, three radioactive peaks were apparent (Figure 9). Identical results were obtained in duplicate experiments. The first peak can be identified as DAP, the second as lysine, the third peak is unknown, although its location corresponds to that of the contaminant in the ( $^3\text{H}$ ) DAP standard. The intensity of all three peaks decreases over time in the lysozyme treated cells. The DAP peak appeared to decrease faster than the other two. Controls also showed some loss of label, however not as much as lysozyme treated cells. When distribution of counts in each of the three peaks was examined, the proportion of counts in the DAP peak in the lysozyme treated cells was seen to decrease with respect to time whereas it remains high in the controls (Table 5). Labeled lysine amounts varied between experiments but did not show a decrease over time.

When all the various parameters are examined together, loss of  $^3\text{H}$  (total,  $^3\text{H}$  (DAP), and % regeneration seem to follow similar curves (Figure 10). Osmotic sensitivity as measured by cell lysis seems to develop faster than tritium loss. In the controls, some drop in ( $^3\text{H}$ ) DAP, and  $^3\text{H}$  total is also observed. This drop however is not of the same magnitude as lysozyme treated cells.

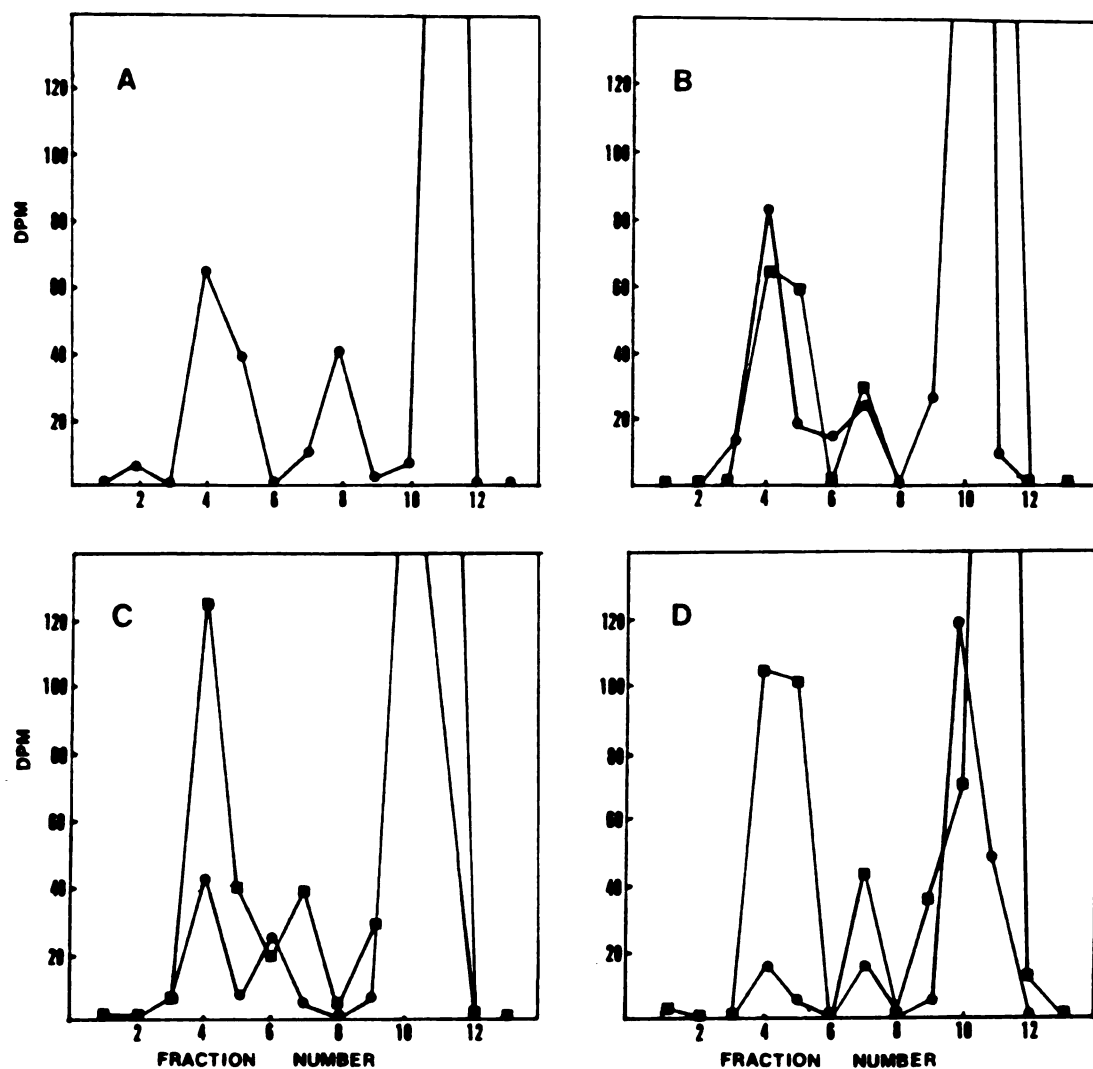


Figure 9. Chromatograms of hydrolysed cells labelled with  $(^3\text{H})$  DAP. Log phase cells were labeled with  $(^3\text{H})$  DAP then treated with 5 mg/ml lysozyme and 0.2 units/ml penicillin. A. Hydrolysed cells prior to lysozyme treatment. B. Hydrolysed cells after 3 hours lysozyme treatment ●, and 3 hour untreated control ■. C. Hydrolyzed cells after 6 hours lysozyme treatment ●, and 6 hour untreated control ■. D. Hydrolyzed cells after 18 hours lysozyme treatment ●, and 18 hour control, ■.

Table 5. Distribution of counts in each peak of hydrolyzed cell chromatograms.

|                | DAP     |         | Lys     |         | Unknown |         |
|----------------|---------|---------|---------|---------|---------|---------|
|                | 1st exp | 2nd exp | 1st exp | 2nd exp | 1st exp | 2nd exp |
| Initial        | 22.5    | 21.7    | 9.77    | .024    | 68.0    | 75.9    |
| 3 hr lysozyme  | 22.3    | 20.9    | 4.93    | .030    | 72.7    | 76.0    |
| 6 hr lysozyme  | 17.3    | 17.6    | 7.87    | .050    | 74.8    | 77.3    |
| 18 hr lysozyme | 10.8    | 8.3     | 8.58    | .023    | 80.5    | 89.4    |
| 3 hr control   | 17.9    | 19.3    | 4.39    | .027    | 77.7    | 78.5    |
| 6 hr control   | 20.6    | 20.7    | 5.13    | .032    | 76.7    | 76.1    |
| 18 hr contro.  | 26.6    | 24.3    | 2.83    | .012    | 70.5    | 74.7    |

Log phase cells were treated with 5 mg/ml lysozyme and 0.2 units/ml penicillin after labeling with ( $^3\text{H}$ ) DAP. Initial cell concentration was 108/ml. % counts in each peak was determined by adding counts in one peak and dividing by total counts in all three peaks. Duplicate experiments were run.

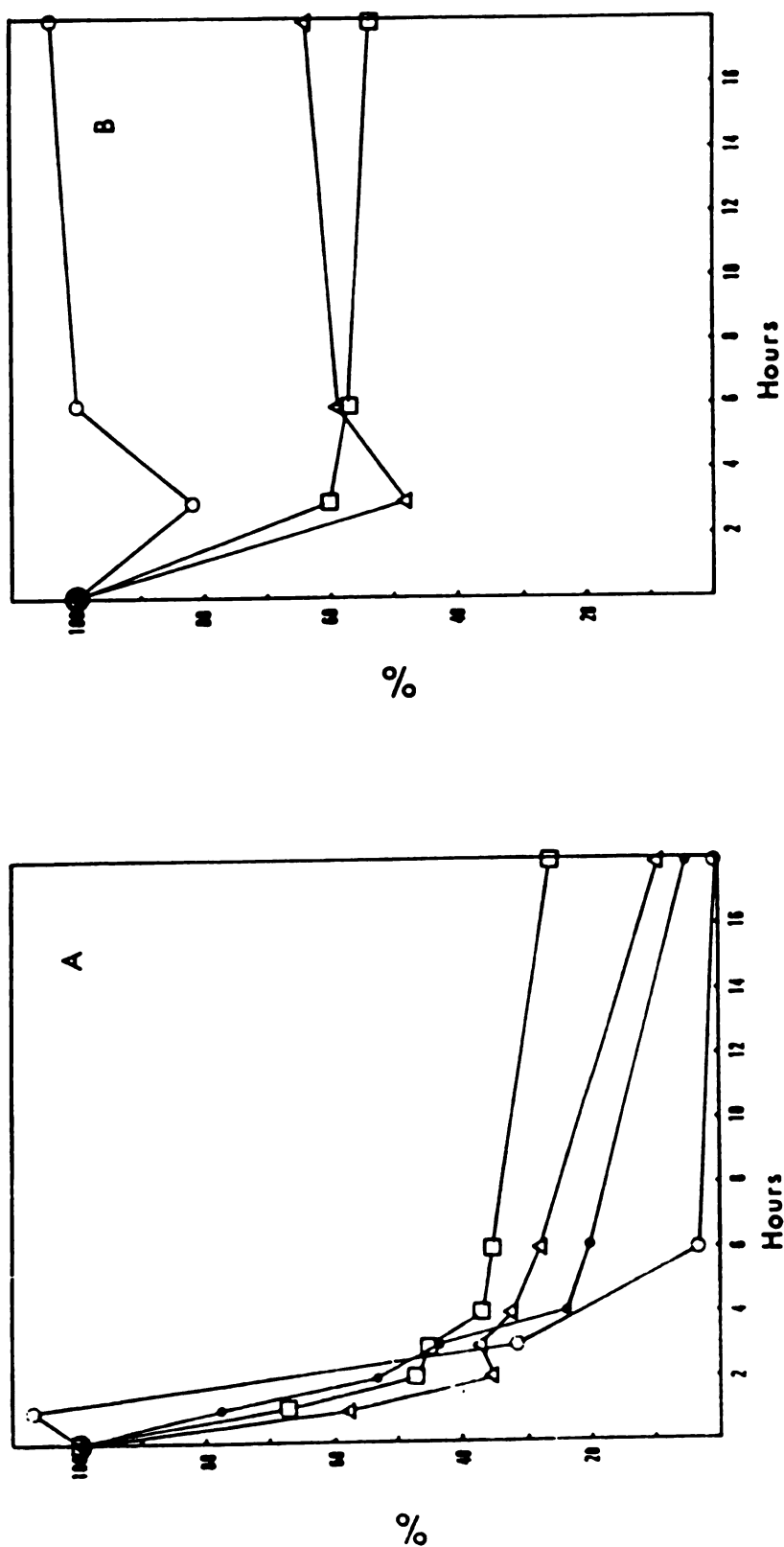


Figure 10. Changes occurring over time during development of protoplasts from ( $^3\text{H}$ ) DAP labelled cells. Log phase cells labelled with ( $^3\text{H}$ ) DAP were treated with 5 mg/ml lysozyme and 0.2 units/ml penicillin. Control samples were run with ( $^3\text{H}$ ) DAP labelled cells resuspended in SMMP without lysozyme. Procedure was as described in materials and methods. Figure 10A: lysozyme treatment. ○-○% whole cells (osmotically insensitive fraction), ●-●%  $^3\text{H}$  DAP (total counts), △-△% ( $^3\text{H}$ ) DAP, □-□% regeneration of protoplasts. Figure 10B: control. ○-○% whole cells, □-□%  $^3\text{H}$  (total counts), △-△% ( $^3\text{H}$ ) DAP.

### Protoplast Fusion

The procedure for fusion of B. lactofermentum was as stated in Materials and Methods. A nonselective regeneration method was utilized because direct selection with antibiotic markers resulted in high background rates. Frequency was determined by dividing the number of colonies that appeared on selective plates by the population added to each plate. Population was determined by plating various dilutions on CM plates.

Initially, two different fusion procedures were compared. The first was based on that described by Kaneko and Sakaguchi (1979) and the second was based on that presented in a patent by Ajinomoto Co., Japan (1983) (Table 6). For the first procedure, 33% PEG at an average m.w. of 6000 plus 5 mM EDTA at pH 7.0 was used. For the second procedure, 33% PEG at an average mw of 6000 plus 10 mM  $\text{CaCl}_2$  at pH 8.0 was used. The data indicated a much higher frequency of recombination for the first procedure than for the second. In fact, the frequency of recombination in the crosses for the second procedure are not substantially different from the control values.

The effect of temperature on fusion and recombination was determined in three separate experiments (Table 7). From these three experiments a trend toward higher frequency rates can be detected with increasing temperature. The highest frequency was obtained at 37°C in Experiment 2.

Table 6. Comparison of two methods for protoplast fusion.

|                                                                       | Frequency of Str <sup>R</sup> Rif <sup>R</sup> |                       |
|-----------------------------------------------------------------------|------------------------------------------------|-----------------------|
|                                                                       | PEG+EDTA                                       | PEG+CaCl <sub>2</sub> |
| Arg <sup>-</sup> Str <sup>R</sup> x Arg <sup>-</sup> Str <sup>R</sup> | 4.1x10 <sup>-7</sup>                           | 1.3x10 <sup>-8</sup>  |
| Met <sup>-</sup> Rif <sup>R</sup> x Met <sup>-</sup> Rif <sup>R</sup> | <1.6x10 <sup>-8</sup>                          | <2.1x10 <sup>-8</sup> |
| Arg <sup>-</sup> Str <sup>R</sup> x Met <sup>-</sup> Rif <sup>R</sup> | 2.8x10 <sup>-6</sup>                           | 1.6x10 <sup>-8</sup>  |

Table 7. Effect of temperature on fusion and recombination frequency.

## Experiment 1

|                                                                      | 25°C                  | 37°C                  |
|----------------------------------------------------------------------|-----------------------|-----------------------|
| Arg <sup>-</sup> Str <sup>R</sup> xArg <sup>-</sup> Str <sup>R</sup> | 7.3x10 <sup>-8</sup>  | 2.8x10 <sup>-7</sup>  |
| Met <sup>-</sup> Rif <sup>R</sup> xMet <sup>-</sup> Rif <sup>R</sup> | <2.3x10 <sup>-8</sup> | <1.2x10 <sup>-8</sup> |
| Arg <sup>-</sup> Str <sup>R</sup> xMet <sup>-</sup> Rif <sup>R</sup> | 1.6x10 <sup>-7</sup>  | 7.4x10 <sup>-7</sup>  |

## Experiment 2

|                                                                      | 25°C                  | 32°C                 | 37°C                  |
|----------------------------------------------------------------------|-----------------------|----------------------|-----------------------|
| Arg <sup>-</sup> Str <sup>R</sup> xArg <sup>-</sup> Str <sup>R</sup> | 5.2x10 <sup>-8</sup>  | 5.8x10 <sup>-8</sup> | 9.4x10 <sup>-8</sup>  |
| Met <sup>-</sup> Rif <sup>R</sup> xMet <sup>-</sup> Rif <sup>R</sup> | <1.4x10 <sup>-8</sup> | 1.1x10 <sup>-8</sup> | <1.7x10 <sup>-8</sup> |
| Arg <sup>-</sup> Str <sup>R</sup> xMet <sup>-</sup> Rif <sup>R</sup> | 2.3x10 <sup>-8</sup>  | 1.8x10 <sup>-6</sup> | 2.8x10 <sup>-6</sup>  |

## Experiment 3

|                                                                      | 37°C                  | 45°C                  |
|----------------------------------------------------------------------|-----------------------|-----------------------|
| Arg <sup>-</sup> Str <sup>R</sup> xArg <sup>-</sup> Str <sup>R</sup> | 1.7x10 <sup>-7</sup>  | 3.6x10 <sup>-8</sup>  |
| Met <sup>-</sup> Rif <sup>R</sup> xMet <sup>-</sup> Rif <sup>R</sup> | <1.9x10 <sup>-8</sup> | <1.9x10 <sup>-8</sup> |
| Arg <sup>-</sup> Str <sup>R</sup> xMet <sup>-</sup> Rif <sup>R</sup> | 1.7x10 <sup>-6</sup>  | 1.1x10 <sup>-6</sup>  |

Frequency of recombination did vary from experiment to experiment even when the same conditions were maintained. However, frequency of crosses were consistently higher than frequency of controls.

Table 8 gives the effect of different average molecular weights of PEG on fusion and recombination frequencies. The highest frequency was obtained when PEG mw 6000 was used. Fusion with PEG mw 1000 did not result in a frequency different from the controls. Length of PEG treatment was also investigated (Table 9). For this experiment PEG mw 6000 was used. No substantial differences were detected over the time range tested. All frequencies were in the same order of magnitude.

Another factor investigated was the effect of protoplast development on fusion and recombination frequency (Table 10). There appears to be a slight increase in frequency after 5 hours lysozyme/penicillin treatment as compared to 2 hours or 16 hours.

When all fusion experiments are examined as a whole, the frequencies of crosses ( $\text{Arg}^- \text{Str}^R \times \text{Met}^- \text{R}_4^R$ ) were in the range of  $10^{-6}$ . The best frequencies ever obtained were about  $10^{-5}$ . These frequencies can be compared to control frequencies of  $10^{-8}$  to  $10^{-7}$ . Frequencies of appearance of the  $\text{Str}^R \text{Rif}^R$  phenotype was  $1.9 \times 10^{-6}$  when an alternate mutant  $\text{Thr}^- \text{Rif}^R$  was used. This is comparable to the frequency obtained with the  $\text{Met}^- \text{Rif}^R$  mutant. Addition of DNase to

Table 8. Effect of different molecular weight PEG on the fusion and recombination frequency.

|                                                                      | Average molecular weight PEG |                       |                       |
|----------------------------------------------------------------------|------------------------------|-----------------------|-----------------------|
|                                                                      | 6000                         | 3000                  | 1000                  |
| Arg <sup>-</sup> Str <sup>R</sup> xArg <sup>-</sup> Str <sup>R</sup> | $6.9 \times 10^{-8}$         | $8.8 \times 10^{-8}$  | $8.3 \times 10^{-8}$  |
| Met <sup>-</sup> Rif <sup>R</sup> xMet <sup>-</sup> Rif <sup>R</sup> | $<2.5 \times 10^{-8}$        | $<2.8 \times 10^{-8}$ | $<2.6 \times 10^{-8}$ |
| Arg <sup>-</sup> Str <sup>R</sup> xMet <sup>-</sup> Rif <sup>R</sup> | $3.0 \times 10^{-5}$         | $7.3 \times 10^{-7}$  | $<3.8 \times 10^{-8}$ |

Table 9. Effect of length of PEG treatment on fusion and recombination frequency.

| Length of Treatment<br>(33% PEG mw 6000) | Frequency of Str <sup>R</sup> Rif <sup>R</sup> |
|------------------------------------------|------------------------------------------------|
| 30 min                                   | $3.0 \times 10^{-5}$                           |
| 15 min                                   | $1.2 \times 10^{-5}$                           |
| 5 min                                    | $2.5 \times 10^{-5}$                           |
| 1 min                                    | $1.2 \times 10^{-5}$                           |

Table 10. Effect of protoplast development on fusion and recombination frequency.

| Length of<br>Lysozyme/<br>Penicillin<br>Treatment | Frequency of Str <sup>R</sup> Rif <sup>R</sup> |                                   |                                   |
|---------------------------------------------------|------------------------------------------------|-----------------------------------|-----------------------------------|
|                                                   | Arg <sup>-</sup> Str <sup>R</sup>              | Met <sup>-</sup> Rif <sup>R</sup> | Arg <sup>-</sup> Str <sup>R</sup> |
|                                                   | Arg <sup>x</sup> Str <sup>R</sup>              | Met <sup>x</sup> Rif <sup>R</sup> | Met <sup>-</sup> Rif <sup>R</sup> |
| 2 hours                                           | $1.2 \times 10^{-8}$                           | $< 1.3 \times 10^{-8}$            | $6.9 \times 10^{-7}$              |
| 5 hours                                           | $3.9 \times 10^{-8}$                           | $< 1.3 \times 10^{-8}$            | $1.2 \times 10^{-6}$              |
| 16 hours                                          | $5.2 \times 10^{-8}$                           | $< 1.1 \times 10^{-8}$            | $4.2 \times 10^{-7}$              |

the fusion procedure had no effect on the frequency of recombination.

## DISCUSSION

B. lactofermentum cells are resistant to treatment with lysozyme. Some method of pretreatment is required in order to obtain good protoplast development. Glycine has been used by others with some success for the development of protoplasts (Baltz, 1978; Hopwood, 1981; Udou et al., 1982). Glycine is thought to replace alanine in the peptidoglycan structure therefore preventing crosslinking and thus resulting in cell walls more susceptible to lysozyme treatment. In these experiments with B. lactofermentum, glycine did not result in good development of protoplasts as determined by microscopic inspection. Therefore penicillin was utilized for development of protoplasts from B. lactofermentum in a procedure similar to that used by Kaneko and Sakaguchi (1979). Care was taken that penicillin at concentrations used for the development of protoplasts was not inhibitory to growth of the organism. An attempt was made to utilize an overnight culture of stationary cells grown in media containing low levels of penicillin. It was hoped that such cells might be more resistant to harsh conditions of treatment and therefore result in higher regeneration rates. Unfortunately, cells treated in this manner also had poor development of

protoplasts as determined by microscopic inspection.

Several different combinations of penicillin treatment concentrations and lysozyme treatment concentrations were examined. The best treatment was the most severe attempted which was 3 u/ml penicillin and 10 mg/ml lysozyme. Higher concentrations were not utilized due to possible inhibitory affects to cell growth. Once penicillin and lysozyme concentrations were established, the effect of media was examined. Growth conditions have been reported to effect not only the development of protoplasts but there regeneration as well (Baltz, 1978). In this study, the growth media and media used to lysozyme treat cells did not display much of an effect on the development of protoplasts. In each case far less than 1% of the cells remained osmotically insensitive. However, the regeneration of protoplasts seemed dramatically affected. Growth in a defined media resulted in regeneration that was 10 fold higher than with growth in a complex media. Regeneration media, by contrast, did not have as dramatic an effect unless protoplasts first grown in complex media were plated for regeneration in a defined media. Such protoplasts exhibited very poor regeneration rates.

Previously published accounts of protoplast development and regeneration in Brevibacterium species indicate that a slight reduction in sucrose concentration resulted in improved development of protoplasts (Kaneko and Sakaguchi, 1979; Shtannikov et al., 1981). However no such effect was

observed in this study. Protoplast development was virtually the same under both concentrations utilized. A slight advantage was detected in regeneration rates when the lower concentration was used, therefore the reduced sucrose concentration was utilized in later procedures.

Initial attempts at protoplast regeneration often resulted in erratic rates even between duplicate samples. It was then discovered that the depth of regeneration agar influenced the rate of regeneration. Maximal regeneration seemed to occur when 10 mls of agar per petri plate was used. It could be hypothesized that less than this amount probably resulted in too much drying at the surface and more may have caused limitation of oxygen to the regenerating protoplasts. It was also hypothesized that accumulation of  $H_2O_2$  might cause the decrease in regeneration rates. Accumulation of  $H_2O_2$  has been shown to affect enumeration of stressed Staphylococcus aureus cells (Flowers et al., 1979). This hypothesis was eliminated when addition of catalase had no effect on regeneration rates.

In the current literature, a number of studies have concluded that the use of BSA can greatly enhance the regeneration of protoplasts (Gabor and Hotchkiss, 1979; Gotz et al., 1981; Minton and Morris, 1983). In this study a similar effect was noted. BSA increased regeneration rates approximately 3 fold in every instance. A possible explanation for this affect is that BSA may act to protect

the proteins at the protoplast surface.

When regenerating protoplasts were examined microscopically, two predominant patterns seemed to emerge. One was a rather large protoplast many times the size of a normal cell. These on occasion had smaller more rod shaped forms in close association with them. More common, however, were smaller protoplasts that seemed to elongate or "bud" with more rod forms adjacent to them. One possible explanation is that the larger forms may be protoplasts that have lost the capacity to revert or divide, therefore merely accumulate more mass.

As noted previously, all optimization studies were run with the wild type strain. When mutants were used, variable results were obtained. Much of the variability in the development of protoplasts could be attributed to growth rates in MM and to somewhat variable penicillin sensitivity as a possible function of growth rate. Two solutions present themselves as methods to eliminate any detrimental effects of this variability. One is to optimize the procedure for each mutant used. The other is to choose mutants with as similar a response as possible to the response of the wild type under optimal conditions developed for the wild type.

Another revelation of the variability in mutant response was that regeneration might correlate to incomplete protoplast development. To further study this relationship the cell

walls of wild type were labeled with ( $^3\text{H}$ ) DAP. These cells were then rendered protoplasts under various conditions and their ability to regenerate evaluated. Although a clear relationship appears to exist between remaining cell wall and regeneration capacity, it is not possible to determine an exact amount of cell wall remaining required to promote regeneration. This was due in part to a large unidentified peak which appeared in chromatograms of cell fractions thus making exact analytical calculation impossible. This unidentified peak corresponded exactly to a very small contaminating peak found in the commercially obtained ( $^3\text{H}$ ) DAP. The peak did not appear to be a breakdown product as further harsh treatment of ( $^3\text{H}$ ) DAP (incubation at  $110^{\circ}\text{C}$  in 6 N HCl) did not result in any change in the proportion of counts found in the contaminating peak. A possible explanation for its increased occurrence in the wild type cell hydrolysates may be that the cells preferentially take up the labeled contaminating compound. Nevertheless, even with the contaminating fraction, an analysis of the % of counts in each peak of chromatogramed samples indicates that DAP is preferentially lost during lysozyme treatment. Some overall label count is also lost. This may be due to some cell lysis or some leaking of intercellular components due to loss of integrity of the cell wall. The total % of labeled lysine also varies between the two experiments shown in Table 5. Although the exact

cause of this variability is unknown, the amount of lysine appears to be unrelated to penicillin/lysozyme treatment.

For fusion of bacterial protoplasts a sequence of events has been hypothesized as stated previously (Ahkong et al., 1975; Frehel et al., 1979; Gumpert, 1980; Knutton and Pasternak, 1979). The first step requires that the protoplasts are brought into close contact with each other. Generally, such close contact is brought about by the use of PEG. The next step is the formation of molecular contacts and alteration of membrane structures. This step may also be enhanced by the action of PEG and may also be affected in some procedures by the addition of divalent cations such as  $\text{Ca}^{2+}$  (Tilcock and Fisher, 1979). The final phases of fusion involve the formation of a fusion membrane and an intermixing of cellular contents. The success of this final phase may depend on removal of PEG (Ferency, 1981; Wojcieszyn et al., 1983).

Ultimately, in order to determine the success or failure of any particular fusion procedure some method must exist to determine if fusion has occurred. In the fusion of eucaryotic cells this method is often visual, making determination of fusion relatively simple. However, for procaryotes, a visual method cannot be utilized due to the much smaller size of the cells involved. As a result, determination of the success of fusion often relies on the appearance of markers, such as antibiotic resistance in the

recombinants. This method of determination is then not a simple measure of fusion, but also of recombination and of the expression of any particular markes. Therefore, fusion expressed as the frequency of appearance of phenotypic markers may actually reflect poorly on the actual frequency of fusion within a population. Nonetheless, it remains the best method available and as such has been used to determine the relative rates of fusion in these studies.

For the fusion of Brevibacterium species protoplasts and protoplasts of closely related organisms, two basic procedures have been described. The first involves the use of 33% PEG plus 10 mM  $\text{CaCl}_2$  at a pH of 8.0. In this study these procedures were compared. The procedure using high concentrations of  $\text{CaCl}_2$  resulted in rates of recombination no higher than that expressed by controls leading to the conclusion that fusion had not occurred under these conditions. This procedure was therefore discarded and future fusions were run using the first procedure.

As mentioned previously, mutant types did express somewhat different behavior than did the wild type when protoplasted and regenerated using the method optimized for the wild type. In addition, it was noted that although conditions were standardized as much as possible, the success or completeness of protoplast development did vary somewhat from experiment to experiment. This variation made it difficult to compare experiments run with different

sets of protoplasts. Therefore, comparisons of frequencies under differing conditions could only be made when the protoplasts used were all of the same lot.

When the effect of temperature on fusion and recombination frequency was examined, three separate experiments were run. Background frequencies varied but never exceeded  $3.0 \times 10^{-7}$ . Recombination frequencies achieved a rate of  $2.8 \times 10^{-6}$  under best conditions. This seemed to occur at  $37^{\circ}\text{C}$ . When the recombination frequency at  $37^{\circ}\text{C}$  was compared to frequencies at other temperatures, the frequency at  $37^{\circ}\text{C}$  was always the highest within any one particular experiment. However, variation of recombination frequency at  $37^{\circ}\text{C}$  between the three experiments was quite large and recombination frequencies at different temperatures (both higher and lower) using a different lot of protoplasts did exceed frequencies at  $37^{\circ}\text{C}$ . This indicates the possible relationship between fusion frequencies and state of development of protoplasts.

The effect of molecular weight of PEG and length of PEG treatment on fusion and recombination frequency was also examined. No clear cut difference could be established between the different time ranges tested. Once again, variability in recombination frequency was high. Molecular weight did appear to affect the frequencies somewhat. However, it should be noted that the various molecular weight ranges of PEG were obtained from different

manufacturers and may have contained variable amounts of impurities. Some researchers have claimed that PEG by itself cannot cause fusion (Honda et al., 1981a). As such, the results obtained here may reflect a difference in the amount of impurity present rather than an actual effect of molecular weight. In addition, it should also be noted that the % PEG at each molecular weight was held constant. One explanation hypothesized for the success of fusion with PEG treatment is that PEG destabilizes membranes by the removal of water (Gibson and Strauss, 1984). This effect may be more pronounced with higher molecular weight PEG when compared to lower molecular weight PEG when the concentration of the lower molecular weight PEG is not increased. Lower molecular weight PEG is more commonly used in eucaryotic cell fusion due to its decreased viscosity which increases the ease of handling of PEG. Concentrations used are generally higher than 33%.

Finally, an attempt was made to determine if the age or developmental state of the product could affect the frequency of recombination. A small effect was observed. Frequencies were highest after 5 hours of lysozyme treatment. This would correspond to a point in time where protoplast development would be maximal. Rates may drop after this period of time due to decreased viability of the protoplasts over time. Prior to 5 hours, there may be too much cell wall remaining, thus interfering with fusion.

## CONCLUSION

Protoplasts can be developed from logarithmically growing cells of B. lactofermentum using a combination of penicillin and lysozyme treatment. The concentrations of penicillin and lysozyme required for good development of protoplasts are 0.3 units and 10 mg per  $10^8$  logarithmically growing cells respectively. The protoplasts developed in this manner can be effectively regenerated in a complex semisoft agar media using a pour plate method. Regeneration of protoplasts is aided by the addition of BSA to the regeneration media.

Optimal regeneration of B. lactofermentum protoplasts appears to be related to the degree of protoplast development. A correlation was found between cell wall remaining and regeneration rates, the less cell wall, the lower the regeneration rates. This could indicate that some fraction of cell wall is required for protoplasts to regenerate effectively.

Protoplasts can be fused effectively using 33% PEG (mw 6000) at  $37^{\circ}\text{C}$ . Temperature does play a role in fusion probably by increasing the fluidity of the membrane. Recombination frequency is, however, highly variable and may depend on the state of development of the protoplasts.

Although fusion can be concluded to have occurred since recombination frequencies under the best circumstances were approximately 10 fold higher than controls, the frequencies obtained were still quite low. As a result, it would not be possible to use this method as developed here for the mapping of genetic markers based on recombination frequencies. However, it does remain a viable method for the exchange of genetic information between various strains and as such will be a useful procedure for strain improvement of industrially useful Brevibacterium strains.

## APPENDIX

## APPENDIX

### Introduction and Literature Review

In conjunction with establishing a possible method to map the chromosome of B. lactofermentum by protoplast fusion, an attempt was made to develop a second alternative method. The method developed was that of nitrosoquanidine (NTG) sequential mutagenesis which is based on work by Ward et al. (1970). NTG sequential mutagenesis first requires the development of a synchronously growing cell culture. This has been accomplished in a variety of ways. Ward et al. (1970) uses nalidixic acid, a gyrase inhibitor, to produce a synchronous culture of E. coli. Others have used (1) size of cell, (2) the tendency of a stationary culture to begin growing again in a synchronous manner (Cutler and Evans, 1966) and (3) nutrient limitation (Kepes and Kepes, 1981; Shehata and Marr, 1970). Once a synchronous culture is achieved, successive samples are treated with NTG (Cerdeña-Olmedo et al., 1968). NTG is believed to cause mutation at the replicating point of the chromosome. Therefore, the type of mutant obtained should reflect the area of chromosome exposed at any specific time. This method has been used in the past to develop a map of E. coli

(Cerdo-Olmedo et al., 1968). The inherent defect of this method is that only a crude map can be established, due to the speed at which the chromosome replicates. For organisms such as E. coli, much better methods such as conjugation are currently available. NTG treatment would be best suited to a slow-growing organism or one in which no other method has been established. For example, it has been used to map just such an organism, Mycobacterium tuberculosis (Woodley et al., 1981). If this method were developed for use in B. lactofermentum it could provide a means of confirming any results obtained by protoplast fusion. It could also provide an alternative method of mapping should protoplast fusion prove to be unsuitable for establishing a map of the chromosome.

### Methods

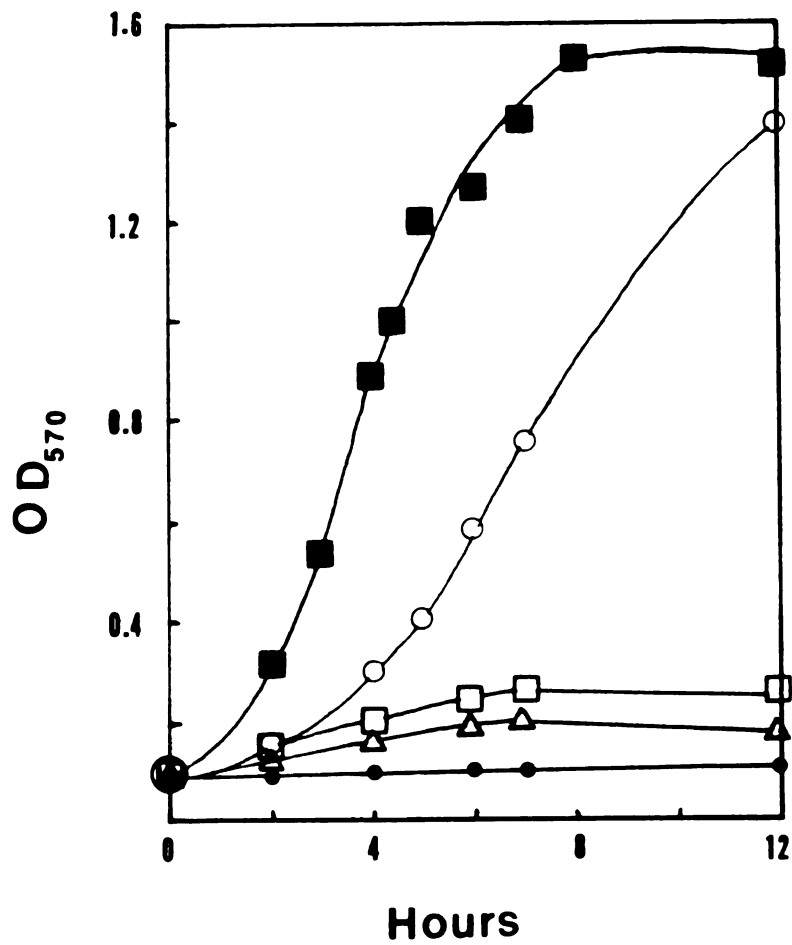
B. lactofermentum was cultivated in CM media or CM agar plates (See Materials and Methods). Overnight cultures were transferred to 5 mls of fresh CM media. After approximately 2 hours, Nalidixic acid was added to a final concentration of 250  $\mu\text{g/ml}$  to synchronize the culture. Cells were removed by filtration and washed with fresh media, then resuspended in fresh media. Fifty  $\mu\text{l}$  of culture was then placed on sterile milipore filters (0.45  $\mu\text{m}$  pore size) on CM plates. For NTG treatment, these filters were placed on plates containing NTG, 0.05 M Tris-maleate or 0.1 M

potassium phosphate buffer (pH 5.5) and 0.45% Difco agar. After appropriate time treatment periods, filters were placed on plates with 0.1 M potassium phosphate (pH 9.0) and 0.45% Difco agar for 5 minutes to destroy residual NTG. Filters with cells were then transferred to CM agar plates for 1 hour to allow expression of mutation, then finally transferred to selective media plates. Selective media plates were MM or CM plus streptomycin or rifampicin (See Materials and Methods).

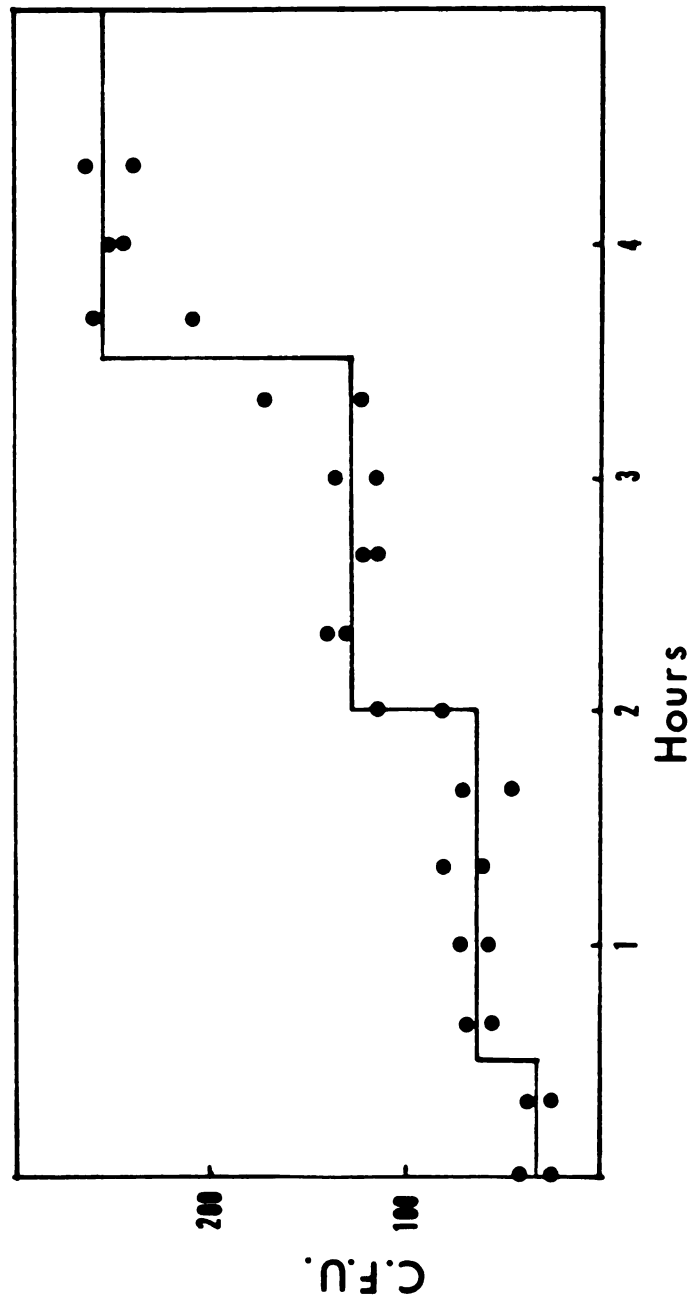
### Results

The optimal concentration nalidixic acid (Nal) was determined as that which completely inhibited growth of B. lactofermentum (Figure 1). For B. lactofermentum this was determined to be 250  $\mu\text{g/ml}$ . This concentration was found to successfully result in synchronous growth as shown by viable cell counts over time (Figure 2).

The amount of NTG required and the length of treatment required to induce reverse mutation of a  $\text{his}^-$  mutant of B. lactofermentum by a filter rotation method was determined as shown in Tables 1 and 2 and in Figure 3. A concentration of 50  $\mu\text{g/ml}$  NTG in the agar for 2 minutes or less seemed to give best results. It was necessary to destroy residual NTG on the filters by brief exposure to agar at pH 9.0. The length of this treatment was determined as shown in Table 3. The effect of this treatment on cell viability



Appendix Figure 1. Effect of NAL on the growth of strain 2256. Cultivation was carried out in CM supplemented with various concentrations of NAL at 30°C with shaking. ●● 250 µg/ml NAL. ▲▲ 125 µg/ml NAL. □□ 75 µg/ml NAL. ○○ 50 µg/ml NAL. ■■ 0 µg/ml NAL.



Appendix Figure 2. Increase in viable cells of strain 2256 after NAL treatment. Cultivation was as in Figure 1. After treatment of cells with 250  $\mu\text{g/ml}$  NAL for 4 hours, NAL was removed by centrifugation and washing of cells with MM devoid of nitrogen. Cultivation was reinitiated at a cell combination of  $1.3 \times 10^8/\text{ml}$ .

Appendix Table 1. Effect of NTG concentration on mutation frequency and cell viability in the membrane filter rotation method.

| Concentration<br>of NTG ( $\mu\text{g/ml}$ ) | No. of his <sup>+</sup><br>per filter <sup>a</sup> | No. of surviving<br>cells per<br>filter <sup>b</sup><br>( $\times 10^5$ ) | Frequency<br>of his <sup>+</sup><br>reverse<br>mutation<br>( $a/10^5$ b) |
|----------------------------------------------|----------------------------------------------------|---------------------------------------------------------------------------|--------------------------------------------------------------------------|
| 0                                            | 9 $\pm$ 4                                          | 275 $\pm$ 6                                                               | $3.3 \times 10^{-7}$                                                     |
| 25                                           | 72 $\pm$ 7                                         | 236 $\pm$ 15                                                              | $3.1 \times 10^{-6}$                                                     |
| 50                                           | 234 $\pm$ 11                                       | 121 $\pm$ 9                                                               | $1.9 \times 10^{-5}$                                                     |
| 100                                          | 12 $\pm$ 3                                         | 3 $\pm$ 2                                                                 | $4 \times 10^{-5}$                                                       |

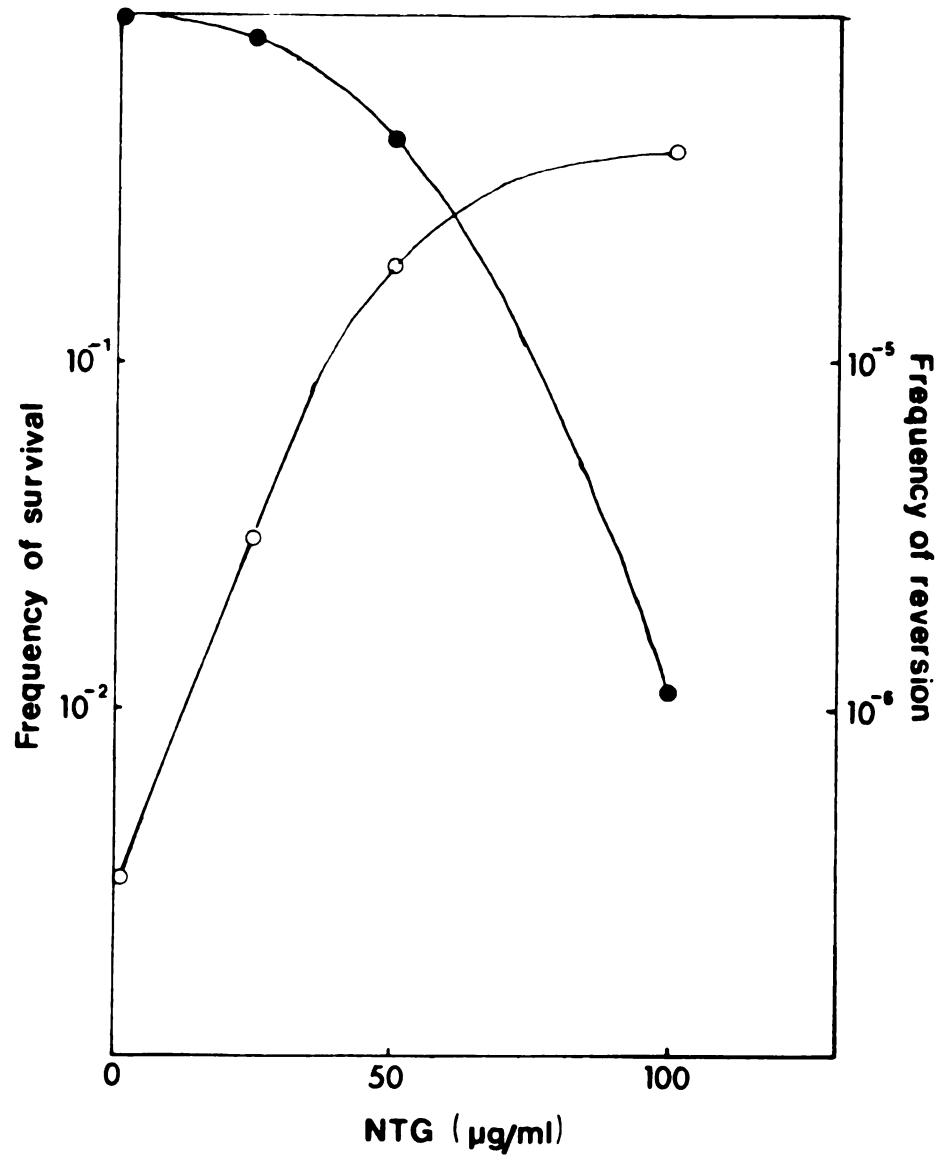
Experimental procedure: see text.

a,b: Mean (in duplicate)  $\pm$  standard error.

Appendix Table 2. Induction of his<sup>+</sup> reverse mutation in his-161 using membrane filters and NTG containing plates (100 µg/ml)

| Placement time of membrane filter on NTG plate (min) | Number of his <sup>+</sup> colonies per membrane filter |
|------------------------------------------------------|---------------------------------------------------------|
| 1                                                    | 53                                                      |
| 2                                                    | 27                                                      |
| 4                                                    | 3                                                       |
| 8                                                    | 0                                                       |
| Non-NTG treatment (control)                          | 0                                                       |

Experimental procedure: see text.



Appendix Figure 3. Effect of NTG concentration on mutation frequency and cell viability in the membrane filter rotation method.

Appendix Table 3. Inactivation of residual NTG by placing membrane filters on pH 9 plate after NTG treatment.

| NTG treatment <sup>a</sup> | Condition for NTG<br>inactivation<br>pH                      Period (min) | Number of<br>surviving<br>cells per<br>membrane<br>filters <sup>b</sup> |
|----------------------------|---------------------------------------------------------------------------|-------------------------------------------------------------------------|
| -                          | -                                                                         | -                                                                       |
| +                          | 7.2                                                                       | 3                                                                       |
| +                          | 9.0                                                                       | 2                                                                       |
| +                          | 9.0                                                                       | 3                                                                       |
| +                          | 9.0                                                                       | 5                                                                       |

<sup>a</sup> Membrane filters were placed for 1 min at 25°C on a NTG-soft agar plate containing 200 µg/ml NTG, 0.05 M Tris-maleate buffer (pH 5.5) and 0.45% Difco agar, then transferred on to pH 9 or pH 7.4 soft agar plate containing 0.1 M phosphate buffer and 0.45% agar.

<sup>b</sup> Mean (in duplicate) ± standard error.

is shown in Table 4.

After determination of optimal conditions of treatment, a few short experiments were run to attempt mapping with this method. The results are shown in Figure 4. The time required for replication of the chromosome appears to be approximately 90 minutes. A possible replication map from these experiments is shown in Figure 5.

### Discussion

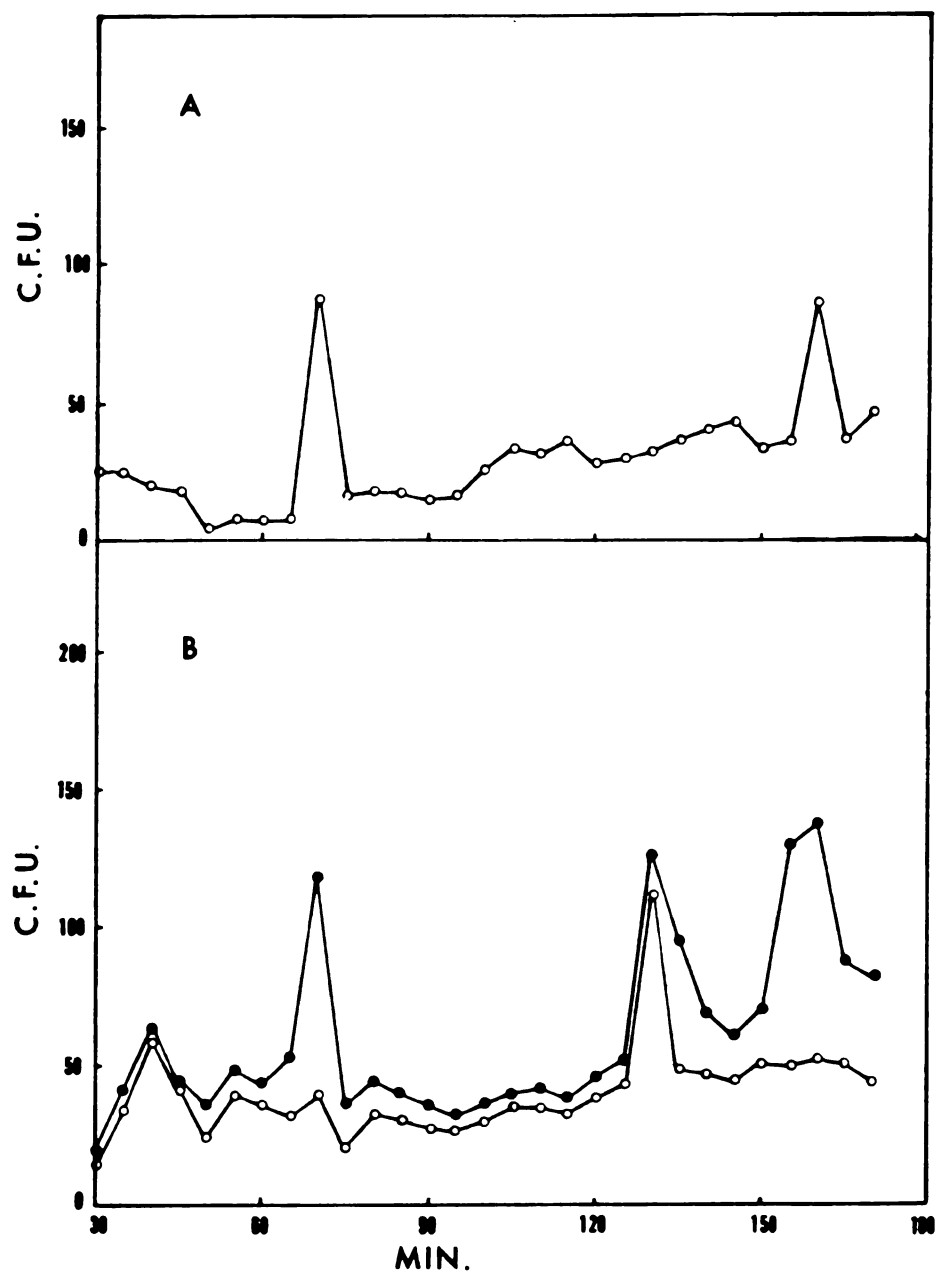
Nalidixic acid provided a convenient method to produce synchronously growing cultures of B. lactofermentum. Figure 1 demonstrates the effect of concentration of Nal on the growth of B. lactofermentum. Because Nal treatment is to be used for a relatively short period of time only to provide synchrony, it was decided that 250  $\mu\text{g/ml}$  Nal would be better than less repressive doses. Figure 2 illustrates the synchrony achieved when growing cells were treated for 4 hours at this concentration of nalidixic acid.

Traditional methods of NTG treatment are somewhat cumbersome and slow. Therefore, a new method was developed to facilitate more rapid and accurate NTG treatment of synchronous cultures. This method involved placing the synchronous cells on filter disks, treating cells with NTG by placing these disks on agar containing NTG and allowing the NTG to diffuse up into the disks. Residual NTG was then denatured by placing the filters plus treated cells on agar

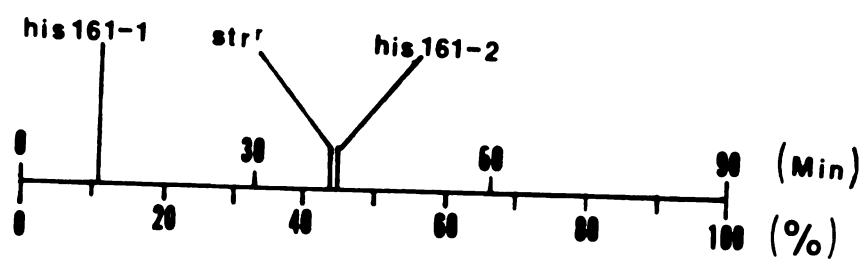
Appendix Table 4. Effect of pH 9 treatment on cell viability.

| Period of treatment<br>(min) | Number of viable cells<br>after treatment |
|------------------------------|-------------------------------------------|
| 0                            | 211                                       |
| 3                            | 223                                       |
| 5                            | 214                                       |
| 15                           | 235                                       |
| 90                           | 218                                       |
| 135                          | 210                                       |

50  $\mu$ l of his 161 cell suspension was applied to each membrane filter, which were then placed on pH 9 plates. At intervals, filters were transferred onto CM-2G plates, followed by incubating them at 30°C for viable cell counting.



Appendix Figure 4. Variation in frequency of streptomycin resistant (A) and histidine reversion (B) mutations.



Appendix Figure 5. Replication map of  $str^R$  and  $his^+$  loci in his 161.

at a very high pH for a short period of time. The effective NTG treatment parameters were determined as shown in Tables 1, 2 and 4. Higher concentration of NTG for shorter time periods seemed most effective. The optimal NTG concentration is described using the parameters as shown in Figure 3. This provides the highest amount of reversion for a particular surviving fraction. In this case the most appropriate concentration is approximately 60  $\mu\text{g/ml}$  NTG. The final treatment for inactivation of NTG was determined as described in Table 2. pH 9.0 for at least 3 minutes was required to denature the NTG as shown by the number of surviving cells. Treatment at this pH was found not to effect cell viability for up to 135 minutes (Table 3).

Figure 4 gives the results from one experiment using NTG treatment on synchronously growing cells. The mutant used was a  $\text{his}^-$  mutant of B. lactofermentum. The generation time under the conditions used was estimated to be 90-100 minutes. Within this generation period, one peak was obtained for streptomycin resistance and 2 peaks were found for reversion to the WT  $\text{his}^+$ . A tentative map of these markers is shown in Figure 5. The second  $\text{his}^+$  peak may represent a suppression mutation. Unfortunately, all data using this method was not as straightforward as that depicted. For the most part, the number of colonies obtained for any particular peak was low and difficult to distinguish from background counts. Initial cell counts

were hard to standardize and may provide a source of error. In addition, standardization of NTG treatment is essential to reproducing results from experiment to experiment and was difficult with the membrane filters.

### Conclusion

The method developed here for NTG treatment of synchronous cultures is faster and more efficient than those described previously. Treatment of membrane filters on agar at pH 9.0 for a period of time to denature the residual NTG is essential to obtaining consistent results. Preliminary data has shown some inconsistency and some problems with low frequency of mutation over background. However, the results are promising. A tentative placement of some markers has been proposed. At this point in time the method may be as useful as protoplast fusion for chromosome mapping.

## BIBLIOGRAPHY

## BIBLIOGRAPHY

- Abe, S. and Takayama, K. 1972. Amino acid-producing microorganisms, variety and classification. In The Microbial Production of Amino Acids. K. Yamada, S. Kinoshita, T. Tounodu and K. Aida, eds. Halsted Press Publishers, 605 Third Ave., N.Y., N.Y. 10016.
- Ahkong, Q.F., Fisher, D., Tampion, W. and Lucy, J.A. 1975. Mechanism of cell fusion. *Nature* 253:194-195.
- Ajinomoto Co., Inc. 1983a. Method for fusion of microbial protoplast. Jpn. Kokai Tokkyo Koho JP 58 158, 181 83,158,181) (Original not seen. Cited from Chem. Abstracts 100:352 (1984)).
- Ajinomoto Co., Inc. 1983b. Fusion of microbial protoplasts. Jpn. Kokai Tokkyo Koho JP 58, 158, 186). (Original not seen. Cited from Chem. Abstracts 100:350 (1984)).
- Akamatsu, T., and Sekiguchi, J. 1982. Transformation of Bacillus protoplasts by plasmid p TP4 DNA. *Agric. Biol. Chem.* 46:1617-1621.
- Akamatsu, T., and Sekiguchi, J. 1983. Selection methods in bacilli for recombinants and transformants of intro- and interspecific fused protoplasts. *Arch. Microbiol.* 134: 303-308.
- Akamatsu, T., and Sekiguchi, J. 1984. An improved method of protoplast regeneration for Bacillus species and its application to protoplast fusion and transformation. *Agric. Biol. Chem.* 48:651-655.
- Arnold, K., Pratsch, L., and Gawrisch, K. 1983. Effect of poly(ethylene glycol) on phospholipid hydration and polarity of the external phase. *Biochim. Biophys. Acta* 728:121-128.
- Asada, M., Nakanishi, K., Matsuno, R. and Kamikubo, T. 1982. Continuous CoA production with immobilized Brevibacterium ammoniagenes cells. *Agric. Biol. Chem.* 46:1687-1688.

- Atlas, R.M. and Bartha, R. 1972. Degradation and mineralization of petroleum by two bacteria isolated from coastal waters. *Biotech. Bioeng.* 14:297-307.
- Baltz, R.H. 1978. Genetic recombination in Streptomyces fradiae by protoplast fusion and cell regeneration. *J. Gen. Microb.* 107:93-102.
- Baltz, R.H. and Matsushima, P. 1981. Protoplast fusion in Streptomyces: Conditions for efficient genetic recombination and cell regeneration. *J. Gen. Microbiol.* 127:137-146.
- Basnak'yan, I., Votrin, I. and Bebow, S.S. 1981. Study of the peculiarities of the action of Brevibacterium ammoniagenes endonucleases on DNA. *Biochemistry* (Engl. trans. *Biokimiya*) 46:1054-1061.
- Bergey's Manual of Determinative Bacteriology, 8th Ed. Williams & Wilkins Publ. Co., Buchanan, R.E. et al., eds. 1974. Part 17, pg. 559, Actinomycetes and Related Organisms.
- Bugaichuk, Y.D., Zuonigorodski, and Zhdanov, V.G. 1981. Conditions for the formation of and reversion to bacillary form of Bacillus licheniformis protoplasts. *Microbiol.* 50:494-497.
- Cerda-Olmedo, E., Hanawalt, P.C. and Guerola, N. 1968. Mutagenesis of the replication point by nitrosoguanidine: Map and pattern of replication of the Escherichia coli chromosome. *J. Mol. Biol.* 33:705-719.
- Chang, S. and Cohen, S. 1979. High frequency transformation of Bacillus subtilis protoplasts by plasmid DNA. *Molec. Gen. Genet.* 168:111-115.
- Cocking, E.C., Davey, M.R., Pental, D. and Power, J.B. 1981. Aspects of plant genetic manipulation. *Nature* 293:265-270.
- Cortzee, J.N., Sirgel, F.A. and Lecatsas, G. 1979. Genetic recombination in fused spheroplasts of Providencia alcalifaciens. *J. Gen. Microbiol.* 114:313-322.
- Collins, M.D., Goodfellow, M. and Minnikin, D.E. 1979. Isoprenoid quinone in the classification of Corneform and related bacteria. *J. Gen. Microbiol.* 110:127-136.

- Crombach, W.H.J. 1971. DNA base composition and DNA homology of Coryneform bacteria isolated from soil, cheese and sea fish. *J. of Gen. Microbiol.* 69:xii.
- Cullis, P.R., and Hope, M.J. 1978. Effects of fusogenic agent on membrane structure of erythrocyte ghosts and the mechanism of membrane fusion. *Nature* 271:672-674.
- Cutler, R.G., and Evans, J.E. 1966. Synchronization of bacteria by a stationary-phase method. *J. Bacteriol.* 91: 469-476.
- Demain, A.L. and Soloman, N.A. 1981. Industrial microbiology. *Scientific American* 245:67-75.
- Demonty, J.A., Robaye, B., and Calberg-Bacq, C.M. 1973. Envelope structure in three different L-forms of Proteus mirabilis. *Antonie van Leeuwenhoek* 39:217-228.
- Eucleigh, D.E. 1981. The microbial production of industrial chemicals. *Scientific American* 245:155-178.
- Ferenczy, L. 1981. Microbial protoplast fusion, in Genetics as a Tool in Microbiology. S.W. Glover and D.A. Hopwood, eds. pp. 1-34, Cambridge University Press.
- Flowers, R.S., Martin, S.E., Brewer, D.G. and Ordal, Z.J. 1979. Catalase and enumeration of stressed Staphylococcus aureus cells. *Appl. Environ. Micro.* 33:1112-1117.
- Fodor, K. and Alföldi, L. 1976. Fusion of protoplasts of Bacillus megaterium. *Proc. Nat. Acad. Sci.* 73:2147-2150.
- Fodor, K. and Alföldi, L. 1979. Polyethylene glycol-induced fusion of bacterial protoplasts: direct selection of recombinants. *Molec. Genet.* 168:55-59.
- Fraley, R.T., Fornairi, C., and Kaplan, S. 1979. Entrapment of a bacterial plasmid in phospholipid vesicles: potential for gene transfer. *Proc. Nat. Acad. Sci.* 76: 3348-3352.
- Frehel, C., Lheritier, A., Sanchez-Rivas, C., and Schaeffer, P. 1979. Electron microscope study of Bacillus subtilis. *Protoplast Fusion* 137:1354-1361.
- Fu, T.T. and Thayer, D.W. 1975. Comparison of batch and semicontinuous cultures for production of protein from mesquite wood by Brevibacterium. *SPJM 98A Biotechnol. Bioeng.* 17:1749-1760.

- Gabor, M.H. and Hotchkiss, R.D. 1979. Parameters governing bacterial regeneration and genetic recombination after fusion of Bacillus subtilis protoplasts. J. Bacteriol. 137:1346-1353.
- Gabor, M.H. and Hotchkiss, R.D. 1982. Analysis of randomly picked genetic recombinants from Bacillus subtilis. Protoplast Fusion. In Genetic and Cellular Technology 1. U.N. Streips, S.H. Goodgal, W.R. Guild and G.A. Wilson, eds. Marcel Dekker Inc., Publ.
- Gerlinas, R.E., Meyers, D.A., Weiss, G.A., Roberts, R.J. and Murray, K. 1977. A specific endonuclease from Brevibacterium albidum. J. Mol. Biol. 114:433-440.
- Gibson, S.M., and Strauss, G. 1984. Reaction characteristics and mechanisms of lipid bilayer vesicle fusion. Biochim. Biophys. Acta 769:531-542.
- Gotz, F., Ahrne, S. and Lindberg, M. 1981. Plasmid transfer and genetic recombination by protoplast fusion by Staphylococci. J. Bacteriol. 145:74-81.
- Guerda, N., Ingraham, J.L. and Cenda-Olmeda, E. 1971. Induction of closely linked multiple mutation by nitrosoguanidine. Nature New Biol. 230:122-125.
- Gumpert, J. 1980. Electron microscopy analysis of protoplast fusion in Streptomyces hydroscopicus and consideration of structural alteration in fusing membranes. Arch. Microbiol. 126:263-269.
- Hales, A. 1977. A procedure for the fusion of cells in suspension by means of polyethylene glycol. Somatic Cell Genetics 3:227-230.
- Honda, K., Maeda, Y., Sasakawa, S., Ohno, H., and Tsuchida, E. 1981a. The components contained in polyethylene glycol of commercial grade (PEG-6,000) as cell fusogen. Biochem. Biophys. Research Commun. 101:165-171.
- Honda, K., Maeda, Y., Sasakawa, S., Ohno, H., and Tsuchida, E. 1981b. Activities of cell fusion and lysis of the hybrid type of chemical fusogens (I) Structure and function of the promoter of cell fusion. Biochem. Biophys. Research Commun. 100:442-448.
- Hopwood, D.A. 1981. Genetic studies with bacterial protoplasts. Ann. Rev. Microbiol. 35:237-272.

- Hopwood, D.A. and Wright, H.M. 1978. Bacterial protoplast fusion: recombination in fused protoplasts of Streptomyces caelicolor. Molec. Gen. Genet. 162:307-317.
- Hopwood, D.A. and Wright, H.M. 1981. Protoplast fusion in Streptomyces: fusions involving ultraviolet irradiated protoplasts. J. Gen. Microbiol. 126:21-27.
- Horvath, R.S. 1971. Cometabolism of the herbicide 2,3,6-trichlorobenzoate. J. Agric. Food Chem. 19:291.
- I'keda, I., Fujita, I. and Yoshinaga, F. 1976. Screening of L-isoleucine producers among ethionine-resistant mutants of L-threonine-producing bacteria. Agric. Biol. Chem. 40:511-516.
- Ingolia, T.D. and Koshland Jr., D.E. 1978. The role of calcium in fusion of artificial vesicles. J. Biol. Chem. 253:3821-3829.
- Ito, T. and Ohnishi, S. 1974.  $\text{Ca}^{2+}$ -induced lateral phase separations in phosphatidic acid-phosphatidyl choline membranes. Biochem. Biophys. Acta. 352:29-37.
- Jones, D. 1975. A numerical taxonomic study of Coryneform and related bacteria. J. Gen. Microbiol. 87:52-96.
- Kaneko, H. and Sakaguchi, K. 1979. Fusion of protoplasts and genetic recombination of Brevibacterium flavum. Agric. Biol. Chem. 43:1007-1013.
- Kao, K.N. and Michayluk, M.R. 1969. A method for high-frequency intergenetic fusion of plant protoplasts. Planta. 115:355-367.
- Kao, K.N., Constabel, F., Michayluk, M.R. and Gamborg, O.C. 1974. Plant protoplast fusion and growth of inter-genetic hybrid cells. Planta 120:215-227.
- Keddie, R.M. and Cure, G.L. 1979. The cell wall composition of distribution of free mycolic acid in named strains of Coryneform bacteria and in isolated from various natural sources. J. Appl. Bacteriol. 42:229-252.
- Kepes, F. and Kepes, A. 1981. Long-lasting synchrony of the division of enteric bacteria. Biochem. Biophys. Research Commun. 99:761-767.

- Knutton, S. and Pasternak, C.A. 1979. The mechanism of cell-cell fusion. *Trends Biochem. Science* 220-223.
- Komagata, K., Yamada, K., and Ogawa, H. 1969. Taxonomic studies on Coryneform bacteria 1: Division of bacteria cells. *J. Gen. Appl. Microbiol.* 15:243-259.
- Kondo, J.K., and McKay, L.L. 1982. Transformation of Streptococcus lactis protoplasts by plasmid DNA. *Appl. Environ. Microbiol.* 43:1213-1215.
- Landman, O.E., and Forman, A. 1969. Gelatin-induced reversion of protoplasts of Bacillus subtilis to the bacillary form: Biosynthesis of macromolecules and wall during successive steps. *J. Bacteriol.* 99:576-589.
- Landman, O.E., Ryter, A., and Frehel, C. 1968. Gelatin-induced reversion of protoplasts of Bacillus subtilis to the bacillary form: Electron-microscopic and physical study. *J. Bacteriol.* 96:2154-2170.
- McDonald, K.O., and Burke, W.F. Jr. 1984. Plasmid transformation of Bacillus sphaericus 1593. *J. Gen. Microbiol.* 130:203-208.
- Minton, N.P., and Morris, J.G. 1983. Regeneration of Clostridium pasteurianum ATCC 6013. *J. Bacteriol.* 155:432-434.
- Momose, H. and Takagi, K. 1978. Glutamic acid production in biotin-rich media by temperature-sensitive mutants of Brevibacterium lactofermentum, a novel fermentation process. *Agric. Biol. Chem.* 42:1911-1917.
- Nakamori, S. and Isamu, S. 1970. Microbial production of L-threonine. Part II. Production by alpha-amino-beta-hydroxyvaleric acid-resistant mutants of glutamate-producing bacteria. *Agric. Biol. Chem.* 34:448-456.
- Nara, T., Misawa, M., Komuro, T. and Kinoshita, S. 1969. Production of nucleic-acid related substances by fermentative processes. Part 21: Biosynthetic mechanisms involved in 5 purine ribonucleotide production by Brevibacterium ammoniagenes. *Agric. Biol. Chem.* 33:358-369.
- Nimi, O., Kubo, M., and Sugiyama, M. 1983. Protoplast formation and the regeneration of Bacillus brevis ATCC 9999 and its mutants. *Biotech. Lett.* 5:709-714.

- Ochi, K., Hitchcock, M.J.M. and Katz, E. 1979. High-frequency fusion of Streptomyces parvulus or Streptomyces antibioticus protoplasts induced by polyethylene glycol. J. Bacteriol. 139:984-992.
- Okamoto, T., Fujita, Y., and Irie, R. 1983. Protoplast formation and regeneration of Streptococcus lactis cells. Agric. Biol. Chem. 47:259-263.
- Papahadjopoulos, D., Poste, G., Schaeffer, B.E. and Vail, W.J. 1974. Membrane fusion and molecular segregation in phospholipid vesicles. Biochem. Biophys. Acta 352: 10-28.
- Papahadjopoulos, D., Vail, W.J., Pangborn, W.A. and Poste, G. 1976. Studies of membrane fusion II. Induction of fusion in pure phospholipid membranes by calcium ions and other divalent metals. Biochem. Biophys. Acta 448: 265-283.
- Peberdy, J.R. 1980. Protoplast fusion - a tool for genetic manipulation and breeding in industrial microorganisms. Enzyme Microbiol. Technol. 2:23-27.
- Pirnik, M.P., Atlas, R.M. and Bartha, R. 1979. Hydrocarbon metabolism by Brevibacterium erythrogenes: normal and branched alkanes. J. Bacteriol. 119:868-878.
- Pitcher, D.G. 1983. Deoxyribonucleic acid base composition of Corynebacterium diphtheriae and other corynebacteria with cell wall type IV FEMS Microbiol. Lett. 16:291-295.
- Pontocorvo, G. 1975. Production of mammalian somatic cell hybrids by means of polyethylene glycol treatment. Somatic Cell Genetics 1:397-400.
- Rose, A.H. 1981. The microbiological production of food and drink. Scientific American 245(3), pp. 127-138.
- Rott, B., Nitz, S. and Freidhelm, K. 1979. Microbial decomposition of sodium pentachlorophenolate. J. Agric. Food Chem. 27:306.
- Rytir, V., Caslavská, J., Konicková-Radochová, M., and Koníček, J. 1982. Modification of the cell wall in Brevibacterium sp. M27. Folia Microbiol. 27:267-268.
- Ryu, D.D.Y., Kim, K.S., Cho, N.Y. and Pai, H.S. 1983. Genetic recombination in Micromonospora rosaria by protoplast fusion. Appl. Environ. Microbiol. 45: 1854-1858.

- Sato, A., and Furuya, A. 1977. Accumulation of guanosine-poly-phosphates by Brevibacterium ammoniagenes; conditions for the accumulation. Agric. Biol. Chem. 41:641-646.
- Schaeffer, P., Cami, B. and Hotchkiss, R.D. 1976. Fusion of bacterial protoplasts. Proc. Nat. Acad. Sci. 73: 2151-2155.
- Schaitman, C.H. 1981. Cell fractionation. In Manual of Methods for General Bacteriology. pp. 52-61, P. Gerhardt, R.G.E. Murray, R.N. Costilow, E.W. Nester, W.A. Wood, N.R. Krieg and G.B. Phillips, eds. American Society for Microb., pub. Washington, D.C.
- Schmidt, R.H., Morris, H.A., Castberg, H.B. and McKay, L.L. 1976. Hydrolysis of milk proteins by bacteria used in cheese making. J. Agric. Food Chem. 24:1106-1113.
- Seiler, H. 1983. Identification key for coryneform bacteria derived by numerical taxonomic studies. J. Gen. Microb. 129:1433-1471.
- Shehata, T.E. and Marr, A.G. 1970. Synchronous growth of enteric bacteria. J. Bacteriol. 103:789-792.
- Shepard, J.F., Bidney, D., Barsby, T. and Kemble, R. 1983. Genetic transfer in plants through interspecific protoplast fusion. Science 219:683-688.
- Shio, I., Sugimoto, S., and Kawamura, K. 1982. Production of L-tryptophan by azaserine-resistant mutants of Brevibacterium flavum. Agric. Biol. Chem. 46:1849-1854.
- Shimizu, S., Tani, Y., and Ogata, K. 1979. Synthesis of CoA and its biosynthetic intermediates by microbial processes. In Methods in Enzymology. McCormic, D.B., and Wright, L.D. eds. Academic Press, N.Y. 62:236-245.
- Shtannikov, A.V., Livshits, V.A. and Zhdanova, N.I. 1981. Fusion of protoplasts and genetic recombination in Coryneform bacteria and in Brevibacterium flavum. Sov. Genet. (Engl. transl. Genetika) 17:924-931.
- Smith, C.L., Ahkong, Q.F., Fisher, D. and Lucy, J.A. 1982. Is purified polyethylene glycol able to induce cell fusion? Biochim. Biophys. Acta 692:109-114.
- Stackebrandt, E. and Fiedler, F. 1975. DNA-DNA homology studies among strains of Arthrobacterium and Brevibacterium. Arch. Microbiol. 120:289-295.

- Stahl, M.L. and Pattee, P.A. 1983a. Computer-assisted chromosome mapping by protoplast fusion in Staphylococcus aureus. J. Bacteriol. 154:395-405.
- Stahl, M.L. and Pattee, P.A. 1983b. Confirmation of protoplast fusion - derived linkages in Staphylococcus aureus by transformation with protoplast DNA. J. Bacteriol. 154:406-412.
- Sun, S.T., Hsiao, C.C., Day, E.P. and Ho, H.T. 1979. Fusion of phosphatidylserine and mixed phosphatidylserine-phosphatidylcholine vesicles: Dependence on calcium concentration and temperature. Biochem. Biophys. Acta. 557:45-52.
- Teshiba, S., and Furuya, A. 1982. Mechanism of 5'-Inosinic acid accumulation by permeability mutants of Brevibacterium ammoniagenes. I. Genetical improvement of 5'IMP productivity of a permeability mutant of B. ammoniagenes. Agric. Biol. Chem. 46:2257-2263.
- Tilcock, C.P.S. and Fisher, D. 1979. Interaction of phospholipid membranes with poly(ethylene glycol)s. Biochem. Biophys. Acta. 557:53-61.
- Tilcock, C.P.S., and Fisher, D. 1982. The integration of phospholipid membranes with poly(ethylene glycol) vesicle aggregation and lipid exchange. Biochim. Biophys. Acta 688:645-652.
- Tosaka, O., and Takinami, K. 1978. Pathway and regulation of lysine biosynthesis in Brevibacterium lactofermentum. Agric. Biol. Chem. 42:95-100.
- Tsuchida, T., Yoshinaga, F., Kubota, K. and Momose, H. 1975a. Production of L-valine by 2-thiazolealanine-resistant mutants derived from glutamic acid-producing bacteria. Agric. Biol. Chem. 39:1319-1322.
- Tsuchida, T., Yoshinaga, F., Kubota, K., Momose, H. and Okumura, S. 1975b. Cultural conditions for L-leucine production by strain no. 218, a mutant of Brevibacterium lactofermentum 2256. Agric. Biol. Chem. 39:1149-1153.
- Udou, T., Ogawa, M. and Mizuguchi, Y. 1982. Spheroplast formation of Mycobacterium smegmatis and morphological aspects of their reversion to the bacillary forms. J. Bacteriol. 151:1035-1039.

- Ward, C.B., Hang, M.W. and Glazer, D.A. 1970. Synchronous reinitiation of chromosome replication in *E. coli* B/r after Nalidixic acid treatment. *Proc. Nat. Acad. Sci.* 66:365-369.
- Weiss, R.L. 1976. Protoplast formation in *E. coli*. *J. Bacteriol.* 128:668-670.
- Wojcieszyn, J.W., Schlegel, R.A., Lumley-Sapanski, K., and Jacobson, K.A. 1983. Studies on the mechanism of polyethylene glycol-mediated cell fusion using fluorescent membrane and cytoplasmic probes. *J. Cell Biol.* 96: 151-159.
- Woodley, C.L., Baldwin, J.N. and Greenberg, J. 1981. Nitrosoguanidine sequential mutagenesis mapping of *Mycobacterium tuberculosis* genes. *J. Bacteriol.* 197: 176-180.
- Wright, W.E. 1978. The isolation of heterokaryous and hybrids by a selective system using irreversible biochemical inhibitors. *Exper. Cell Research* 112:395-406.
- Wyrick, P.B. and Rogers, H.J. 1973. Isolation and characterization of cell wall defective variants of *Bacillus subtilis* and *Bacillus licheniformis*. *J. Bacteriol.* 116:456-465.
- Yamada, Y., Inouye, G., Tahara, Y. and Kondo, K. 1976. The menaquinone system in the classification of Coryneform and Nocardioform bacteria and related organisms. *J. Gen. Appl. Microbiol.* 22:203-214.
- Yamada, K. and Komagatu, K. 1970a. Taxonomic studies on Coryneform bacteria, II. Principal amino acids in the cell wall and their taxonomic significance. *J. Gen. Appl. Microbiol.* 16:103-113.
- Yamada, K. and Komagatu, K. 1970b. Taxonomic studies on Coryneform bacteria, III. DNA base composition of Coryneform bacteria. *J. Gen. Appl. Microbiol.* 16: 215-224.
- Yoshinaga, F. 1969. Studies on the fermentation production of L-proline. IV. Mechanism of L-proline production by *Brevibacterium flavum*. 2247 no. 14-5. *J. Gen. Appl. Microbiol.* 15:387-398.

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