Isolation and Characterization of Mutants Resistant against Chlorate of *Bacillus licheniformis*

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SUMMARY

From *Bacillus licheniformis* S244, 45 *chl* deletion mutants have been isolated which belong to six groups. In four groups the identity of the auxotrophic markers deleted has been established: *his-2*, *ura-1*, *arg-3* and *trp-1*. Furthermore, a *leu* and a *glu* marker were found in the two other groups of deletion mutants. From *B. licheniformis* S1026, derived from strain ATCC9945A, 36 single site mutants were obtained and classified into eight groups, *chl A* to *H*, by transformation and phage SP15-mediated transduction. The *glu* deletion comprises the mutations *chl A* and *B*. In total, 13 *chl* mutations have been found and there may be up to 13 *chl* genes. Of the single-site *chl* mutations, only *chl E* is linked with a known reference marker, *ura-1*. All *chl* mutants are unable to form nitrate reductase under conditions of oxygen shortage. This nitrate reductase is mainly associated with the cytoplasmic membrane. Most mutations have pleiotropic effects: (i) retarded growth under anaerobic conditions; (ii) an increased sensitivity to penicillin and related antibiotics; (iii) completely altered membrane protein patterns on acrylamide-SDS gels.

In the wild-types, 11 membrane protein bands are observed. There are only small, quantitative differences between membrane protein patterns of cells cultivated aerobically or anaerobically. Membrane protein patterns of the *chl* mutants are most different from those of the wild-types after anaerobic cultivation. Differences include appearance of a new protein band, disappearances, decreases, doublings and often large increases of other protein bands. Aerobically, the mutants have membrane protein patterns less different from those of the wild-types. Differences include appearance of a new protein band, decrease and disappearance of protein bands. An explanation for all these changes may be a wholesale disorganization of protoplasmic membrane biosynthesis.

INTRODUCTION

Many bacteria are able to use nitrate instead of oxygen as a terminal electron acceptor under anaerobic conditions. In this process nitrate is reduced to nitrite by the enzyme nitrate reductase. Nitrate reductase also reduces chlorate, and the product chlorite is toxic. Consequently, nitrate reductaseless mutants can be obtained by plating bacteria on chlorate-containing media and incubating anaerobically (Piéchaud *et al.* 1967; Stouthamer, 1967).

In Enterobacteriaceae both deletion and single-site chlorate resistant (*chl*) mutants have been obtained by this method. In *Escherichia coli*, five *chl* loci have been mapped by deletion and linkage mapping methods (Puig & Azoulay, 1967; Adhya, Cleary & Campbell, 1968; Venables & Guest, 1968; Guest, 1969; Puig, Azoulay, Gendre & Richard, 1969; McGregor & Schnaitman, 1971). In *Salmonella typhimurium* seven *chl* loci have been mapped by deletion and linkage mapping (Stouthamer, 1969; Sanderson, 1970; Stouthamer

& Bettenhausen, 1970) and in *Klebsiella aerogenes* three *chl* loci have been mapped by deletion analysis alone (Stouthamer & Pietersma, 1970).

Chlorate-resistant mutants show different pleiotropic effects depending upon the bacterial species concerned. In *Escherichia coli* *chl* mutants, in addition to the formation of nitrate reductase, the formation of formic hydrogenase is affected (Guest, 1969; Ruiz-Herrera, Showe & de Moss, 1969). In *Proteus* and *Salmonella* effects are found on the formation of reductase for thiosulphate, tetrathionate and chlorate (Piéchaud *et al.* 1967; de Groot & Stouthamer, 1969; Stouthamer, 1969). All these enzymes are membrane bound. The pleiotropic effect is explained by the hypothesis that some structural component of the membrane-bound multi-enzyme complex has been lost or altered, so that not only nitrate reductase but also other enzymes cannot be complexed into the membrane in an active form. In *E. coli* *chl* mutants alterations in the membrane protein composition have been observed (Schnaitman, 1969; Onodera, Rolfe & Bernstein, 1970; McGregor & Schnaitman, 1971).

For the study of chlorate-resistant mutants of a Gram-positive organism we have chosen *Bacillus licheniformis* because it is a denitrifying species; much of the regulation of nitrate reductase formation is known (Schulp & Stouthamer, 1970), and it is accessible to genetic analysis by transformation (Gwinn & Thorne, 1964) and transduction (Taylor & Thorne, 1963).

In the present paper the isolation and genetic classification of *chl* single site and deletion mutants will be described. Some of these mutations have been mapped. An analysis of the membrane protein composition of the mutants will be presented, together with some additional characterization of the mutants.

METHODS

Bacterial strains. The parent strains and relevant markers are listed in Table 1. Except s244, all strains were derived from ATCC 9945A. Strains were maintained on slopes of Oxoid Brain Heart Infusion agar (BHI) and stored at 4 °C. They were transferred to fresh agar slopes every 3 weeks; asporogenous strains every 4 days. BHI agar for thymine auxotrophs was fortified with 10 µg/ml thymine. The map positions of the reference markers have been determined by Tyeryar, Lawton & McQuillan (1968), Rogolsky (1970) and McCuen & Thorne (1971).

Media. NBY, NBYG, NBYGNO₃ and NBYNO₃ have been described previously (Schulp & Stouthamer, 1970). If solid media were required 2% Oxoid agar no. 1 was added; for semisolid media 0.5% agar was used. NBYGChl was prepared by addition of 6.25 g/l of KClO₃ to NBYG. The minimal medium was that of Thorne & Stull (1966), without sodium glutamate. Growth requirements for auxotrophs were added according to Thorne & Stull (1966). Minimal plates for *thy* auxotrophs contained 100 µg/ml thymine; for glutamic acid auxotrophs sodium glutamate 200 µg/ml was used. For scoring *spo* colonies the minimal medium was fortified with two 10⁻⁵ M-MnSO₄ (Rogolsky, 1970). TM and BLSG media for transformation were prepared according to Thorne & Stull (1966). PA medium for transduction was prepared according to Thorne (1962). Later a modified medium PA was used (Taylor & Goldberg, 1971). PA medium containing 0.1% (w/v) glucose was used to grow *chl* mutants.

Cultural conditions. All solid media were incubated at 37 °C. NBYGChl plates were incubated anaerobically under nitrogen and the anaerobic jar was flushed three times with nitrogen. To score nitrate utilization on NBYNO₃ plates, the plates were incubated under nitrogen containing less than 10 p.p.m. oxygen; the jar was flushed seven times with this

Table 1. Parent strains and strains with reference markers of *Bacillus licheniformis*

Strain no.	Markers	Original designation	Origin
S244	—	430	J. Woldendorp, Wageningen
S292	—	ATCC 9945A	C. B. Thorne, Fort Detrick
S1026	<i>str pep-1</i>	FD01	J. D. Sherratt, Edinburgh
S1168	<i>ura-1</i>	FD08	M. Rogolsky, Utah
S1169	<i>pep-1 pur-1 leu-2 his-2</i>	FD19	
S1170	<i>pep-1 met-4</i>	M3R-1	
S1171	<i>pep-1 lys-8</i>	M2R-1	
S1177	<i>spo LR 26</i>	LR 26	
S1179	<i>spo LR 9</i>	LR 9	
S1328	<i>pep-1</i>		S1177 $\xrightarrow{td}$ S1171
S1329	<i>pep-1 leu-2 his-2</i>		S1177 $\rightarrow$ S1169
S1333	<i>pep-1 arg-3</i>	M18R-1	M. Rogolsky, Utah
S1337	<i>pep-1 str-1 thi-1</i>	MR-1	
S1338	<i>pep-1 pur-1</i>	M17R-1	
S1339	<i>pep-1 trp-1</i>	FD09	
S1340	<i>pep-1 gly-3</i>	M28	
S1393	<i>pep-1 ile</i>		From S1328 by NG treatment
S1397	<i>pep-1 thy</i>		As above, followed by aminopterin enrichment
S1404	<i>pep-7 ade-2</i>		C. B. Thorne, Amherst, Massachusetts

Abbreviations used: *ura*, uracil; *pur*, purine; *leu*, leucine; *his*, histidine; *met*, methionine; *lys*, lysine; *arg*, arginine; *thi*, thiamine; *trp*, tryptophan; *gly*, glycine; *ile*, isoleucine; *thy*, thymine; *ade*, adenine requirement; *pep*, inability to form glutamyl polypeptide; *spo*, inability to sporulate; *str*, resistance to streptomycin; $\xrightarrow{td}$, obtained by SP 15-mediated transduction.

gas. A mixture of 1 g kieselguhr, 0.1 g pyrogallol and 0.1 g NaHCO₃ wrapped in paper were present in the jar to remove the last traces of oxygen.

Batch cultures were grown at 37 °C in a New Brunswick Micro Ferm Laboratory fermentor in a 5 l jar containing 2 l of medium with vigorous aeration (10 l air/min, agitation 650 rev./min) or weak aeration (2 l air/min, agitation 200 rev./min) or anaerobically (nitrogen, agitation 100 rev./min). The medium in the jar was inoculated with 3 × 100 ml of culture in NBY, shaken overnight at 250 rev./min at 37 °C in a 500 ml bottle. The medium was maintained at pH 7 by a New Brunswick Automatic pH Controller using 0.5 N-NaOH. Bacterial density was measured by extinction at 660 nm (*E*₆₆₀) in a Unicam SP 600 spectrophotometer.

Isolation of auxotrophic mutants. Auxotrophic mutants were isolated from S1328 by an *N*-methyl-*N*-nitroso-*N*-guanidine (NG) treatment (Rogolsky, 1969). Thymine auxotrophs were isolated after aminopterin enrichment following NG mutagenesis (de Haan, Stout-hamer, Felix & Mol, 1963).

Propagation and assay of phage SP15. Phage SP15 was propagated on *Bacillus subtilis* w23 *str* by an overlay technique similar to that described by McCuen & Thorne (1971). Sufficient moisture was maintained by packaging the Petri dishes in a polythene bag with a piece of dripping wet cotton-wool. In this way, phage titres of 2.5 to 5 × 10⁹ p.f.u./ml were obtained. Phage was assayed in a similar manner.

Transduction procedure. Donor lysate preparation and transduction procedure were those of Rogolsky (1970). A 3 ml sample of phage lysate and 3 ml of acceptor cells were used, concentrated and spread on to minimal medium plates supplemented where necessary with nutritional requirements, or on to NBYNO₃ plates. This yielded 50 to 500 colonies/plate. Modified PA medium (Taylor & Goldberg, 1971) gave better results.

Testing recombinants for chl markers. In most crosses *chl* was the unselected marker. Recombinants were tested for chlorate resistance by streaking on to NBYGChl plates and incubating anaerobically. Chlorate-resistant recombinants showed dense and 'closed' growth like the *chl* donor strains; chlorate-sensitive recombinants like the chlorate-sensitive acceptor strains produced only a few colonies.

Nitrate and nitrite reduction. Nitrate and nitrite reduction were tested qualitatively by inoculating 5 ml of NBYGNO₃ or NBYG containing 0.01 % NaNO₂. After overnight or 2 day incubation at 37 °C, presence of nitrite was checked with Boehringer-Mannheim nitrite test strips.

Testing for antibiotic sensitivities. Bacteria were grown in NBY overnight on the shaker at 37 °C and 250 rev./min; 0.1 ml was spread on to Oxoid BHI agar plates and sensitivity discs were placed on the plates. The diameter of inhibition zones was measured after incubation at 37 °C overnight. The discs used were: chloramphenicol, 10 µg; methicillin, 10 µg; colistin, 50 µg; fusidic acid, 10 µg; kanamycin, 5 µg; neomycin, 10 µg; novobiocin, 5 µg; cloxacillin, 5 µg; ampicillin, 5 and 25 µg; gentamycin, 10 µg; cephaloridin, 25 µg; colistin methane sulphonate, 200 µg; sulphafurazole, 500 µg; carbenicillin, 100 µg; sulfa-methoxazole trimethoprim, 25 µg; tetracycline, 50 µg.

Isolation of DNA. DNA was isolated according to Gwinn & Thorne (1964). No recrystallization in saline citrate was performed. The final concentration of the DNA solution obtained was 70 µg/ml.

Transformation procedure. The transformation method was that of D. J. Sherratt (personal communication). Recipient cells were shaken overnight at 37 °C in BLSG medium and then diluted 20-fold with TM medium. To 0.9 ml of this cell suspension 0.1 ml of suitably diluted DNA was added and the mixture shaken for 2 h, then 9 ml TM without glucose was added and the suspension centrifuged at 3000 rev./min. The cells were then washed twice with 10 ml of TM without glucose resuspended in 0.5 ml TM without glucose and 0.2 ml of the suspension was spread on to NBYNO₃ plates which were incubated anaerobically. TM without glucose for washing and resuspending prevented glucose supported anaerobic growth on NBYNO₃ plates.

Isolation of peripheral membranes and mesosomes. Bacteria were cultivated in batch cultures to an E660 of 1.0 or until the beginning of the stationary phase if that occurred earlier. They were harvested by centrifugation at 10000 g, at 2 °C for 15 min. Peripheral membranes and mesosomes were isolated according to Reaveley & Rogers (1969). Both peripheral membranes and mesosomes from 1 l of culture were resuspended in 4 ml of 1 M-NaCl+0.02 M-MgSO₄ and used for enzyme assays.

Preparation of samples for polyacrylamide gel electrophoresis. The membrane pellet from 1 l of culture was homogenized in 4 ml of 0.01 M-sodium phosphate buffer, pH 7.0, with 1 % sodium dodecyl sulphate (SDS) and 1 % mercaptoethanol, incubated at 37 °C for 2 h and centrifuged at 24 °C at 18000 rev./min. The supernatant was dialysed overnight against 1 l of 0.01 M-sodium phosphate buffer with 0.1 % SDS and 0.1 % mercaptoethanol.

Polyacrylamide gel electrophoresis. Gel electrophoresis was performed according to Weber & Osborn (1969) on 7 cm gels with 7 mA/gel for 5 h. By that time the bromphenol blue front had migrated 65 mm. The amount of protein applied varied from 50 to 200 µg/gel. Gels were stained in a 1 % amido black 10B solution in 7 % acetic acid; surplus of stain was removed by rinsing 24 to 48 h in a gently stirred 7 % acetic acid solution. Gels were stored in 7 % acetic acid and scanned on a Kipp densitometer.

Molecular weights were calculated from the R_m values according to Weber & Osborn

(1969). A standard curve, made with seven proteins (pyruvate kinase, glutamate dehydrogenase, aldolase, alcohol dehydrogenase and trypsin) was in good agreement with that of Weber & Osborn (1969).

Electron microscopy. Membrane pellets were fixed according to Ryter & Kellenberger (1958*a*), dehydrated in graded solutions of acetone and embedded in Vestopal-A (Ryter & Kellenberger, 1958*b*). Thin sections were cut on an LKB Ultratome with glass knives, placed on formvar and carbon copper grids and stained with uranyl acetate (Stempak & Ward, 1964). Negatively stained specimens were prepared by the method of Parson (1963) using 2% ammonium molybdate, pH 7.5, as a staining solution. Electron micrographs were taken with a Philips EM 300 electron microscope operated at 80 kV using a 50 μ m objective aperture.

Ribosomal proteins. Purified ribosomes were a gift of Mr Joh. Brouwer, Biochemisch Laboratorium der Vrije Universiteit. They had been prepared according to van Dijk-Salkinoja, Stoof & Planta (1970). The pellet was dissolved in phosphate-SDS-mercaptoethanol buffer and treated as the membrane preparations.

Other techniques. Preparation of sonic extracts and measurement of acid formation have been described previously (Schulp & Stouthamer, 1970).

Oxygen uptake was measured by standard Warburg techniques. Cytochrome spectra were recorded as difference spectra between oxygen oxidized and dithionite reduced cell suspensions on an Aminco Chance Double Beam Spectrophotometer.

Nitrate reductase activity was measured according to Lowe & Evans (1964); the assay mixtures were incubated for 15 min at 37 °C. Protein was estimated according to Lowry, Rosebrough, Farr & Randall (1951).

RESULTS

Isolation of chlorate-resistant mutants. *Bacillus licheniformis* s244 was shaken overnight at 37 °C in NBY, diluted 1:10, shaken for a further 4 h and diluted 50- and 100-fold. Samples of each dilution (0.1 ml) were plated on to 40 NBYGChl plates and incubated anaerobically at 37 °C for 2 days. For selection of *chl* deletion mutants plates were replicated on to minimal medium and NBY. After incubation at 37 °C potential auxotrophs were isolated, their deficiencies were identified and they were characterized more closely (see Table 2).

In 15 experiments the percentage of deletion mutants among the chlorate-resistant mutants was 0.15%. Deletion mutants never reverted to chlorate sensitivity or prototrophy. It was impossible to transduce these mutants either to prototrophy or chlorate sensitivity although they could act as donors in SP15-mediated transduction crosses.

No deletion mutants were obtained in this way from the transformable strain S1026 but 36 single-site chlorate-resistant mutants were obtained which had a low rate of reversion.

Genetic classification of the single-site mutants. Twenty of the 36 *chl* mutants were readily transformable. They have been classified by reciprocal transformation crosses into six groups designated *chl A* to *F* (Table 3). The remaining 16 mutants were used as donors in transformation crosses with representatives of groups *chl A* to *F* as receptors. Thirteen of the 16 mutants were classified into groups *chl A*, *D* and *E*. The three remaining strains which did not belong to one of the previous groups have been classified into two different groups *chl G* and *H* by reciprocal SP15-mediated transduction.

Relationship between single-site and deletion mutations. To study whether the deletions overlapped the single-site mutations, donor DNA and SP15 donor lysates were prepared from all the deletion mutants. Representatives of the groups *chl A* to *H* were used as

Table 2. *Chlorate resistant deletion mutants of Bacillus licheniformis* S244

Experimental details are given in Results (Isolation of chlorate-resistant mutants).

Type	No. of experiments	No. of mutants obtained
del (<i>ura chl I</i>)	6	20
del (<i>leu chl J</i>)	8	12
del (<i>arg chl K</i>)	2	5
del (<i>trp chl L</i>)	2	4
del (<i>glu chl A chl B nir</i>)	2	3
del (<i>his chl M</i>)	1	1

Table 3. *Genetic classification of the single site chl mutants from Bacillus licheniformis* S1026

Recombinants were scored on NBYNO₃ plates which were incubated anaerobically as described. In each experiment controls were run to score revertants and for competence or transducibility.

Group	Method employed			Total no.
	Reciprocal transformation	Donor capacity in transformation	Reciprocal transduction	
<i>chl A</i>	5	1	—	6
<i>chl B</i>	2	—	—	2
<i>chl C</i>	1	—	—	1
<i>chl D</i>	1	3	—	4
<i>chl E</i>	9	9	—	18
<i>chl F</i>	2	—	—	2
<i>chl G</i>	—	—	2	2
<i>chl H</i>	—	—	1	1
Total no.	20	13	3	36

Table 4. *Identification of the auxotrophic genes deleted in chl deletion mutants of Bacillus licheniformis* S244

SP15 lysates were prepared from donor cells and tested for transducing capacity. The acceptors were transduced with these lysates and with an S1026 lysate to check their transducibility. Recombinants were scored on minimal medium plates, if necessary fortified with growth factors.

Donor	Acceptor	Selected marker	Recombinants
S937 del (<i>trp chl L</i>)	S1339 <i>trp-1</i>	<i>trp</i>	—
S948 del (<i>leu chl I</i>)	S1329 <i>leu-2 his-3</i>	<i>leu</i>	+
S950 del (<i>arg chl K</i>)	S1333 <i>arg-3</i>	<i>arg</i>	—
S959 del (<i>his chl M</i>)	S1329 <i>leu-2 his-3</i>	<i>his</i>	—
S1005 del (<i>ura chl J</i>)	S1168 <i>ura-1</i>	<i>ura</i>	—

acceptors in transduction and transformation crosses conducted as described in the previous section. The *glu* deletion comprises the genes *chl A* and *B*. All other deletion and single site mutations are different. Possibly therefore 13 genes may be involved in chlorate resistance: the genes *A* to *H* and the genes in the *ura*, *leu*, *arg*, *trp* and *his* regions designated *chl I*, *J*, *K*, *L* and *M* respectively.

Identification of the auxotrophic markers in the deletions. In *Bacillus licheniformis* a number of auxotrophic markers have been mapped. To identify the auxotrophic genes in the deletion mutants, SP15 donor lysates were prepared from the deletion mutants and auxotrophic mutants with markers at known map positions were transduced with these lysates (Table 4). We conclude that the *leu* mutation in the deletion is not the *leu-2* gene. The *ura*, *his*, *arg* and *trp* genes in the deletions are *ura-1*, *his-3*, *arg-3* and *trp-1* respectively.

Fig. 1. Chromosome map of *Bacillus licheniformis* after Tyeryar *et al.* (1968), Rogolsky (1970) and McCuen & Thorne (1971), showing the position of some *chl*-markers. Solid lines indicate linkage in SP15-mediated transduction crosses. Dashed lines indicate absence of linkage; their length is arbitrary. The approximate positions of *chl*-markers, found by deletion mapping, are indicated by arrows. The numbers represent distances in linkage units between two markers (calculated at 100 % co-transduction).

Chl J, K, L and *M* are therefore in the *ura-1*, *his-3*, *arg-3* and *trp-1* regions. In Fig. 1 their approximate map position has been indicated by arrows. Localization of the *chl A* and *B* genes was not possible because a single site *glu* mutant was not available.

Mapping *chl* markers by SP15-mediated transduction. SP15 donor lysates were prepared from *chl* strains and all auxotrophic strains listed in Table 1 were used as acceptors in transduction crosses. Prototrophic recombinants were scored and tested for *chl* properties. Linkage of *chl* and *spo* markers was studied using the *spo* strains as donors and the *chl* strains as acceptors. Chlorate-sensitive recombinants were scored on NBYNO₃ plates and then tested for *spo* properties.

Chl E is 43 % linked to *ura-1* but unlinked to either *spo-B* or *leu-2* the markers on either side of *ura* with which *ura* is not linked. Consequently the order *ura-1-chl E* relative to the replication origin cannot be determined (Fig. 1).

The other *chl* markers were not located, most likely because they are outside the mapped regions of the *Bacillus licheniformis* chromosome.

Nitrate and nitrite reduction in *chl* mutants. None of the *chl* mutants was able to grow anaerobically with nitrate as a sole electron acceptor. No nitrite, nor reduced benzyviologen-nitrate oxidoreductase was produced after a shift from aerobic to anaerobic conditions. Trace amounts of nitrite were formed after prolonged incubation with nitrate.

No mutant was affected in nitrate and nitrite assimilation with the possible exception of the *glu* deletion mutants. These mutants require glutamic acid in the medium which acts as a repressor of the assimilative nitrate reductase (Cove, 1966, Downey & Nuner, 1967) so that nitrate assimilation cannot be tested.

Only the *glu* deletion mutants and one *chl A* mutant s1279 are unable to reduce nitrite (*nir*). This is not a pleiotropic effect of the *chl A* mutation as the other *chl A* mutants are *nir*⁺, but it is caused by a separate mutation in the same genetic region. s1279 should be

Fig. 2. Growth rates of *Bacillus licheniformis* wild-type and mutants after a shift from aerobic to anaerobic conditions. Δ , s244 wild-type in NBYGNO₃; $\circ$, s244 wild-type in NBYG; $\bullet$, s950 del (*arg chl K*) in NBYG and NBYGNO₃; $\times$, s950 del (*his chl M*) in NBYG and NBYGNO₃.

Table 5. Acid formation of *Bacillus licheniformis* s244 wild-type and some *chl* mutants after anaerobic cultivation in NBYG

Averages of two experiments are given.

Strain ...	s244	s970	s959	s950	s1005
Acid formation ($\mu\text{mol/mg protein/h}$)	11.1	12.9	15.1	8.9	21.7

considered a deletion mutant, because it bears two mutations, does not revert and cannot act as an acceptor in crosses.

Glaser & de Moss (1971) have observed restoration of nitrate reductase activity in *chl D* mutants of *Escherichia coli* by addition of 10^{-4} M-molybdate. We did not observe this effect in any of the *Bacillus licheniformis chl* mutants.

Growth characteristics of chl mutants. In strain s244 wild-type anaerobic growth with glucose is stimulated by the addition of nitrate (Schulp & Stouthamer, 1970).

Anaerobic growth of mutants is not stimulated by addition of nitrate and all mutants except *ura* deletion mutants show retarded growth under anaerobic conditions; some do not reach a higher extinction than *E660* = 0.8 (Fig. 2). Their glycolytic activities show some variation but from these figures it is clear that retarded fermentation cannot account for the retarded growth (Table 5).

When the mutants are cultivated with weak aeration, their growth rate decreases as soon

Table 6. *Protein and nitrate reductase content of cell fractions*

The membrane fraction contained 2300 units of nitrate reductase after osmotic shock and centrifugation. Three hundred and eighty units are lost during washing with 1 M-NaCl + 0.02 M-MgSO₄.

	Protein [mg/l of culture (<i>E</i> 660 = 1.0)]	Nitrate reductase [Units/l of culture (<i>E</i> 660 = 1.0)]	Nitrate reductase (sp. activity, units/mg protein)
Crude sonicate	185	2400	13
Membranes	37	1920	52
Mesosomes	2.4	17.6	7.3
Supernatant of mesosomes	N.D.	0	0

N.D., not determined.

as the oxygen supply becomes limiting. Even under these conditions no nitrate reductase or nitrite is formed.

These results indicate that pleiotropic effects of *chl* mutations in *Bacillus licheniformis* are very different from those in Enterobacteriaceae, where growth rates are not influenced by *chl* mutations (Piéchaud *et al.* 1967; Stouthamer, 1967).

Aerobically, growth rate, oxygen uptake with different substrates, and cytochrome spectra of mutants did not differ from those of the wild-type.

Antibiotic sensitivity of chl mutants. The two parent strains and all types of *chl* mutants were tested for their sensitivities to a selection of antibiotics and chemotherapeutics. All the single-site mutants, *chl A* and *F* excepted, had an increased sensitivity to penicillin and its structural analoga ampicillin, methicillin, carbenicillin and cephaloridin. All these antibiotics interfere with cell wall synthesis.

Localization of nitrate reductase in the cell. Strain s244 wild-type was cultivated anaerobically in NBYGNO₃ to *E*660 = 1.0. From 1 l of culture membrane and mesosome fractions were prepared. From 100 ml of the culture a crude sonic extract was prepared. Protein content and nitrate reductase activity of these preparations were determined (Table 6). Nearly all nitrate reductase is located in the membranes. Specific and total nitrate reductase activities in the mesosomes are insignificant. Therefore, only the protein composition of the membranes has been studied.

The *c*-type cytochrome, $\alpha = 553$, was also located in the membranes.

Contamination of the membrane and mesosome fractions. Membranes from *Bacillus licheniformis* isolated according to Reaveley & Rogers (1969) contained little or no cytoplasmic proteins. The main contamination was RNA, especially when isolation was performed in the presence of 0.02 M-MgSO₄ and it could not be completely removed. Similar observations have been found by Yudkin & Davis (1965), Coleman (1969) and van Dijk-Salkinoja *et al.* (1970).

Electron micrographs of thin sections of *Bacillus licheniformis* s244 membranes revealed the presence of vast amounts of ribosomes (Fig. 3), so ribosomal protein bands are to be expected in acrylamide gels of the membrane fraction. Flagella were present in the mesosome fraction, not in the membrane fraction as observed in negatively stained specimens.

Distinction between ribosomal and membrane proteins. Attempts were made to achieve a separation of ribosomal and membrane proteins. For this purpose, the membranes were solubilized by the method of van Dijk-Salkinoka *et al.* (1970). In this way a mixture of insoluble components, ribosomes and solubilized membrane protein was obtained. The purified membrane proteins were isolated by differential centrifugation and subjected to

Fig. 3. Thin sections of the membranes of *Bacillus licheniformis* s244 at two different magnifications. Included ribosomes are clearly visible. There is a slight contamination with cell wall material. Scale bars equal 5 μm .

Fig. 4. Absorbance scans of polyacrylamide gels of *Bacillus licheniformis* s244 membranes (—) and ribosomes (---). Below the scan is a representation of the gel. Some bands which appear as one peak on the scans are actually two very close bands (6, 6a, 11, 11a). Hatching indicates diffuse bands.

Fig. 5. Absorbance scan of a polyacrylamide gel of *Bacillus licheniformis* s244 membrane proteins, after anaerobic cultivation in NBYGNO₃. Below the scan is a representation of the gel. Arrows indicate differences with membrane proteins of cells cultivated aerobically in NBYGNO₃. Only bands 1 up to 13 are shown.

Table 7. Molecular weights of the membrane proteins of *Bacillus licheniformis*

Molecular weight of proteins were determined electrophoretically by the method of Weber & Osborne (1969).

No.	Molecular weight (daltons)	No.	Molecular weight (daltons)
1	133 000	7*	59 000
2	110 000	8	55 000
3	96 000	9	48 000
4	86 000	10	43 000
5	78 000	11 + 11 a	37 000
5 a*	70 000	12	32 000
6 + 6 a	66 000		

* Detectable only in *chl*-mutants.

polyacrylamide gel electrophoresis as described. Only four protein bands were detected in the purified membrane protein fraction. All cytochromes remained in the insoluble fraction. Complete separation of ribosomes and membrane proteins was impossible, therefore the ribosomal proteins have been identified by comparing polyacrylamide gels of purified ribosomes and membranes (Fig. 4). The protein bands found in wild-type and mutant membranes have been numbered from 1 to 18 but one protein band may consist of a mixture of several proteins.

Protein bands 1 to 12 were hardly or not at all found in the ribosomes, but the ribosomes were enriched for protein bands 13 to 18 compared with the membranes. The maximum

Table 8. *Comparison of the membrane protein patterns of chl mutants with the wild-type after anaerobic cultivation in NBYGNO₃*

	Protein band in which change is observed						
	1	2	3	5	6	7	8
S1005 del (<i>ura chl J</i>)	+	++	o	o	o	p	o
S937 del (<i>trp chl L</i>)	o	o	o	d	o	p	o
S948 del (<i>leu chl I</i>)	o	o	o	d	o	p	—
S950 del (<i>arg chl K</i>)	a	++	o	d	—	p	o
S959 del (<i>his chl M</i>)	o	o	o	d	o	p	a
S970 del (<i>glu chl A chl B nir</i>)	o	o	—	d	o	p	o
S1182 <i>chl A</i>	o	+	o	o	o	o	o
S1186 <i>chl B</i>	o	++	—	o	o	o	o

a, Absent in the mutant but present in the wild-type.

p, Present in the mutant but absent in the wild-type.

d, Doubling of band.

+, Increase.

++, Large increase.

—, Decrease.

o, No change from wild-type.

molecular weight of ribosomal proteins, apart from a minor component of mol.wt 65000, varies according to different data from 28300 to 33000 (Dzionara, Kaltschmidt & Wittman, 1970; Garrett, Rak, Daya & Stöffler, 1971). Protein bands 1 to 12 are therefore considered as true membrane proteins (Table 7). Bands 13 to 18 consist mainly of ribosomal protein but some membrane proteins may to be present which are completely masked by ribosomal proteins.

It was impossible to detect nitrate reductase on these gels, as solubilization of the membranes with 1% SDS causes a complete inactivation of nitrate reductase.

Influence of cultural conditions upon the membrane protein composition of wild-type. S244 was cultivated aerobically and anaerobically in NBYGNO₃. Membranes were isolated and their protein composition was analysed. There are only a few quantitative differences between the membrane protein pattern of aerobic and anaerobic cells (Fig. 4, 5). The most pronounced effects are increases of bands 8 and 10.

Alterations in membrane protein composition of chl mutants after anaerobic cultivation. A selection of the mutants were cultivated anaerobically and their membrane protein composition was analysed. As the mutants had been derived from two different parent strains, these parent strains S244 and S1026 were compared for their membrane protein patterns and no differences were found.

Each mutant has its own characteristic and reproducible pattern of membrane protein bands. Most of the differences, however, occurred in more than one type of mutant. Changes were observed in the amounts of protein in bands 1, 2, 3, 5, 6, 7 and 8 (see Table 8).

A very pronounced effect is the large increase in the amount of protein band 2. This is shown in Fig. 6 for S950 del (*arg chl K*). There is a doubling of band 5, a decrease in the amount of protein in band 6 and the appearance of band 7.

The absence of protein band 8 in S959 del (*his chl M*) is shown in Fig. 7 as well as the presence of band 7 and doubling of band 5.

Alterations in membrane protein composition of chl mutants after aerobic cultivation. Effects of *chl* mutations were produced mainly under anaerobic conditions. Aerobically,

Fig. 6. Absorbance scan of a polyacrylamide gel of *Bacillus licheniformis* s950 del (*arg chl K*) membrane proteins, after anaerobic cultivation in NBYGNO₃. The arrows indicate differences with s244 wild-type cultivated under the same conditions. Only bands 1 up to 13 are shown.

Fig. 7. Absorbance scan of a polyacrylamide gel of *Bacillus licheniformis* s959 del (*his chl M*), membrane proteins after anaerobic cultivation in NBYGNO₃. The arrows indicate differences with s244 wild-type cultivated under the same conditions. Only bands 1 up to 13 are shown.

Fig. 8. Absorbance scan of a polyacrylamide gel of *Bacillus licheniformis* s950 del (*arg chl K*) membrane proteins, after aerobic cultivation in NBYGNO₃. The arrows indicate differences with s244 wild-type cultivated under the same conditions. Only bands 1 up to 13 are shown.

Table 9. Comparison of the membrane protein patterns of *chl* mutants with the wild-type, after aerobic cultivation in NBYGNO₃

	Protein band in which change is observed				
	1	2	6	7	8
s948 <i>chl J</i>	o	o	o	p	o
s950 <i>chl K</i>	a	a	—	p	o
s937 <i>chl L</i>	a	a	o	p	o
s959 <i>chl M</i>	a	a	—	p	a
s970 <i>chl A chl B</i>	a	a	o	p	o

Symbols as in Table 8.

no effects of *chl* mutations were observed. It seemed worth while to investigate the membrane protein composition of the *chl* mutants after aerobic cultivation to distinguish constitutive and inducible differences.

Membrane protein composition of s1005 del (*ura chl J*), the single site mutants and the wild-type are identical. All other deletion mutants have protein band 7 which is lacking in the wild-type. Other differences, the absence of protein bands 1 and 2 and decrease of protein band 6, occur in some other mutants.

Protein band 8 is lacking in s959 del (*his chl M*) both aerobically and anaerobically — an important characteristic of this mutant. The differences in membrane protein composition are summarized in Table 9. To show the large difference between the membrane protein pattern after aerobic and anaerobic cultivation the densitogram of s950 membrane protein from aerobic cells is shown in Fig. 8.

DISCUSSION

Deletion mapping has proved extremely useful in mapping *chl* loci of several Enterobacteriaceae (Adhya *et al.* 1968; Stouthamer, 1969; Stouthamer & Pietersma, 1970). In these organisms deletions comprising up to eight genes have been found (Stouthamer, 1969).

The auxotrophic *chl* mutants obtained from *Bacillus licheniformis* s244 are considered

as deletion mutants for the following reasons: (i) they cannot revert nor be transduced or transformed to either prototrophy or chlorate sensitivity; (ii) mutants of the same type have been isolated repeatedly in independent experiments; (iii) mutagenic treatment yields only single auxotrophs at a very low frequency. In contrast, without mutagenic treatment, plating on NBYGChl is a selective method only, yet *chl*-auxotrophic double mutants were obtained at high frequency; (iv) the *glu* deletion comprises four genes: *glu*, *chlA*, *chlB*, *nir*; (v) the *chl* genes deleted are all different as they cause different membrane protein patterns.

Most deletions comprise only one *chl* and one auxotrophic gene; this may be a consequence of the fact that the chromosome of *Bacillus licheniformis* is less well marked than that of *Escherichia coli*.

The number of single site *chl* mutations, eight, seems high and one might wonder whether these mutations are in eight different genes. Both *chl A* and *chl B* are situated near *glu*, yet they are phenotypically different. *Chl E* is the only gene mapping near *ura*. The five remaining mutations have not been mapped. *Chl F* is phenotypically different from the rest (penicillin sensitivity). For the mutations *chl C*, *D*, *G* and *H* the possibility cannot be ruled out that they are within the same gene or cistron. However, van Hartingsveldt, Marinus & Stouthamer (1971) employed a similar technique for classification of *Pseudomonas aeruginosa chl* mutants. These mutations have been mapped by conjugation (van Hartingsveldt & Stouthamer, 1973) and they are at different sites on the chromosome and therefore in different genes. Further mapping may reveal similar data for the *Bacillus licheniformis chl* mutations as well.

Bacillus licheniformis chl mutants differ markedly from Enterobacteriaceae, *chl* mutants in their pleiotropic effects. In Enterobacteriaceae pleiotropic effects are limited to effects upon reductases and formic dehydrogenase complex; all of them are properties connected with anaerobic electron transport. In *B. licheniformis chl* mutations cause (i) absence of nitrate reductase under conditions of oxygen shortage, (ii) decrease in growth rate under anaerobic conditions, (iii) increased sensitivity to penicillin and related antibiotics, (iv) very complex changes in membrane protein patterns mainly under anaerobic but also under aerobic conditions. In some of the single-site mutants all these changes are found. It may be concluded that these changes are due to the pleiotropic effects of one mutation since revertants to chlorate sensitivity behave like wild-type in all these properties.

We believe that the increased penicillin sensitivity in some mutants is due to a pleiotropic effect of *chl* mutations on cell wall synthesis, which is a membrane-bound process (Araki *et al.* 1966; Izaki, Matsushashi & Strominger, 1966, 1968). Evidence exists both for *Escherichia coli* and *Bacillus subtilis* that the targets for penicillin are situated on the membrane (Cooper, 1956; Lawrence & Strominger, 1970; Hartman, Hölte & Schwarz, 1972).

The changes in membrane protein patterns produced in *Bacillus licheniformis* by the *chl* mutations are very complex and important compared with changes produced in *Escherichia coli* by such mutations as *chl*, *tol*, *dna* (Inouye & Pardee, 1970; Samson & Holland, 1970; Shapiro, Siccaldi, Hirota & Jacob, 1970; McGregor & Schnaitman, 1971). In all these cases the main differences are observed in one or two protein bands only. In contrast, *B. licheniformis chl* mutants cultivated anaerobically have important alterations in two to five protein bands.

The explanation for all these changes may be a wholesale disorganization of protoplasmic membrane biosynthesis. Consequently, it is hard to say whether the pleiotropic effects are consequences of the chlorate resistance or the chlorate resistance a consequence of, for instance, the antibiotic sensitivity. In this respect symbol *chl* may be considered a phenotypic, not a genotypic designation for some of the mutants.

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