Alterations in the Cytoplasmic Membrane Proteins of Various Chlorate-Resistant Mutants of *Escherichia coli*

C. H. MacGregor and C. A. Schnaitman

Department of Microbiology, University of Virginia School of Medicine, Charlottesville, Virginia 22901

Received for publication 21 June 1971

Chlorate-resistant mutants corresponding to each known genetic locus (chlA, chlB, chlC, chlD, chlE) were isolated from *Escherichia coli* K-12. All these mutants showed decreased amounts of membrane-bound nitrate reductase, cytochrome b, and formic dehydrogenase, but all had normal succinic dehydrogenase activity. Proteins from the cytoplasmic membranes of these mutants were compared to those of the wild type on polyacrylamide gels. The addition of nitrate to wild-type anaerobic cultures caused increased formation of three membrane proteins. These same proteins, along with one other, were missing in varying patterns in mutants altered at the different genetic loci. One of the missing proteins was found to be the enzyme nitrate reductase, although this protein was present in some mutants lacking nitrate reductase activity. None of the others has been identified.

The cytoplasmic membrane of *Escherichia coli* is the site of organization of several enzyme systems. One of these, the electron transport system involving the membrane-bound enzymes nitrate reductase and formic dehydrogenase, has recently received much attention due to the isolation of chlorate-resistant mutants. These mutations involve a pleiotropic loss of both of these enzymes (4). Chlorate-resistant mutants are easily isolated by the addition of KCIO₃ to the anaerobic growth medium, and they have been mapped at five different genetic loci (8). The pleiotropic nature of these mutations has been explained as resulting from alterations in the structure of the membrane on which these enzymes are localized or in the mechanism assembling these particulate components (4, 5). Because loss of the nitrate system is not harmful to the cell, and a wide range of mutants can easily be obtained, these chlorate-resistant mutants provide an excellent means of studying the synthesis, assembly, and regulation of such particulate systems. By a procedure recently developed in this laboratory (15), the cytoplasmic membranes of these mutants, freed from contaminating cell wall, were separated into their protein components, and relative amounts of each component were examined.

**MATERIALS AND METHODS**

**Media.** Standard minimal salts were previously described (14). Carbon sources (sodium succinate, Casamino Acids, or glucose) were autoclaved separately and added to a final concentration of 1%. When anaerobic growth was desired, cultures were grown in sealed flasks, with a mixture of 95% N₂ and 5% CO₂ bubbled continuously through the culture. Cultures were labeled with ³H-leucine and ³H-tyrosine as previously described (14). Complex medium contained 30 g of tryptose soy broth per liter. Where indicated, 0.1% KNO₃ was added to either minimal salts or complex medium. Chlorate medium contained 30 g of tryptose soy broth, 15 g of agar, 2 g of glucose, and 2 g of KClO₃ per liter. (Glucose and chlorate were autoclaved separately.) L-medium and L-agar for transduction were those of Lennox (9). Soft agar contained 8 g of nutrient broth, 5 g of NaCl, and 6.5 g of agar per liter. Medium selective for amino acid markers was minimal salts with 15 g of agar per liter, to which thiamine and amino acids were added to a final concentration of 0.01%, adenine to 0.004%, and glucose to 1%. Transduction medium for λ contained 10 g of tryptose per liter and 5 g of NaCl per liter. EMB agar contained 27.4 g/liter of Levine EMB agar without lactose, plus 1% galactose.

**Strains.** To isolate chlorate-resistant mutants, RK20 was grown in broth to log phase, and samples of this culture were plated on chlorate-agar and incubated in a Brewer jar under H₂ for 24 hr at 37 C. Single colonies were picked and purified by restreaking on chlorate-agar. The chlorate genes are designated as in Taylor (18) with the exception that narE (Taylor) is referred to as chlE for simplicity.

Phage P1kc was obtained from R. Kadner. Phages λ⁺, λำ, and λ₁₂₂ were obtained from R. Huskey.
Transduction. Transduction methods of Lennox (9) were employed for phage P1kc. Lysates of donor strains were prepared with confluent lysis plates, and phage multiplicities of two were used. After adsorption of phage, samples of the adsorption mixtures were plated on media selective for amino acid markers. When selecting for Bio+ transductants, adsorption mixtures were pelleted and suspended in minimal salts and glucose with 0.5% sodium citrate and incubated for 45 min at 37°C before plating on minimal salts with glucose. In each case, transductants were picked from the selective plates and restreaked onto the same medium before examination for cotransduction on chlorate agar. Lysates of λdg were prepared by inducing a culture of RK20(λ) which originated from a single colony transduced by λ+ from Gal+ to Gal-. Immunity to λ was tested by infection of the transductants with λcl&y, and λcl&y was shown by all of the transductants to be sensitive (13). For transduction, λ-broth cultures of the recipient strains were grown to about 10⁸ cells/ml. A 0.2-ml amount of this culture was mixed with 0.1 ml of the λdg lysates, incubated for 15 min at 23°C, and diluted 100-fold. Samples were plated on EMB agar. Gal+-transductants were picked and restreaked on EMB agar before examination for cotransduction on chlorate-agar.

Enzyme assays and cytochrome measurements. For all measurements, cells were broken by passage through a French pressure cell, and the envelope fraction was isolated as previously described (14). This was suspended in the appropriate buffer and used as a crude particulate preparation or solubilized by extraction with Triton X-100. This procedure was shown to preferentially separate the cytoplasmic membrane proteins from the Triton-insoluble cell wall (15). Formic dehydrogenase and nitrate reductase assays were previously described (13). Both of these assays involve the use of artificial electron donors and acceptors, thus insuring that low activity cannot be due to the lack of a cytochrome. Enzyme preparations, identical except for the addition of Triton to 2%, gave very close values when nitrate reductase was measured; apparently this enzyme is not affected by the presence of 1 to 2% Triton. Succinic dehydrogenase was measured by coupling the enzyme via phenazine methosulfate to the reduction of cytochrome c (2). This was followed spectrophotometrically at 550 nm. Each sample was added to 2.5 ml of a mixture containing 100 ml of 0.1 m phosphate buffer (pH 7.6), 5 ml of 1 m sodium succinate, (pH 7.4), 1.5 ml of KCN (5.8 mg/ml), 20 ml of 10⁻⁴ m ethylenediaminetetraacetic acid (pH 7.4 to 7.6), and 85 ml of water. This was allowed to incubate at 23°C for 15 min before 1.95 ml was transferred to a cuvette and 15 μl of cytochrome c (104 mg/ml) was added. To start the assay, 25 μl of phenazine methosulfate (12 mg/ml) was added. Cytochrome c used was Sigma type III. Cytochrome b measurements were made in a Cary double-beam spectrophotometer with a 0.1 OD slide wire.

Polyacrylamide gel electrophoresis. Except for the purified nitrate reductase, all material separated on the gels was cytoplasmic membrane protein solubilized by Triton extraction (15). The solubilized proteins were freed of lipid and Triton by passage through a Sephadex LH-20 column equilibrated with acidified dimesitylformamide, and electrophoresis was carried out as previously described (14, 15) in sodium dodecyl sulfate-containing gels. Whatman DE52 was used to purify nitrate reductase for electrophoresis.

RESULTS

Identification of chlorate-resistant mutants. Chlorate-resistant mutants were isolated from strain RK20. At least one mutant of each type was selected from the five known genetic loci affecting the enzyme nitrate reductase. Positions on the E. coli linkage map of the mutations to chlorate resistance were first determined by transduction mapping with P1kc (Table 2). Phage P1 lysates were made from each of the mutants and used to infect two recipient strains, RK1009 and W3350. When Met+ was selected, only mutant 36 showed any linkage, and therefore it was designated chiB. Selection for Trp+ showed that mutation 19 was closely linked and was therefore in the chiC region. Because chiC mutants retain formic dehydrogenase activity, they can be easily identified by looking for gas production in a fermentation tube. (Other chlorate-resistant mutants are Gas-). For this reason, the single Gas+ mutant, 19, was checked for linkage to trp; mutant 34 was checked as a control, and other Gas- mutants were not tested. Mutants 16 and 20 gave the same cotransduction frequencies, and, as they were most closely linked to the bio locus, they were designated chlA. Mutants 13, 44, and 3 were all considerably farther from bio and thus were called chiE. Although mutant 34 also gave a cotransduction frequency with bio which was similar to that of the chiE mutants, it was shown to be on the opposite side of the bio gene by mapping with phage λ. Each mutant was also infected with a lysate from RK20 (λ gal+). This lysate contained a high proportion of λdg phage carrying the gal+ gene as well as everything on the E. coli chromosome between gal and the λ phage attachment site. Since this includes chID, all λdg stocks should carry chID+, and thus all mutants transduced to Gal+ should also be transduced to chlorate sensitivity if the mutation is in the chiD region. Only mutant 34 could be transduced to chlorate-sensitive by infection with λdg. This mutant was picked from a group of chlorate-re-
sistant mutants possessing nitrate reductase levels which were detectable, although much lower than the wild-type level. According to Adhya et al. (1), one characteristic of the chlD locus is retention of partial nitrate reductase activity, which is present even when the locus is entirely deleted.

**Enzyme assays and cytochrome measurements.**

It is known that the synthesis of the enzyme nitrate reductase is increased by the addition of nitrate to the growth medium (16). When grown anaerobically in minimal salts with Casamino Acids and glucose, wild-type *E. coli* produced a low amount of this enzyme (Table 3). Upon the addition of nitrate to the medium, production increased approximately sevenfold. In the mutants, nitrate reductase levels were extremely low or undetectable with or without the addition of nitrate. As predicted by the nature of the chlD locus, mutant 34 contained the highest nitrate reductase activity. It has been shown that, in *E. coli*, electron transport to nitrate reductase involves cytochrome *b*, and that the amount of cytochrome *b* increases upon the addition of nitrate to the anaerobic growth medium (12). Because this cytochrome *b* is also presumably bound to the cell membrane, it was of interest to us to look at the amount of cytochrome *b* in membranes of the mutants. The mutants all possessed cytochrome *b*, but in amounts lower than

<table>
<thead>
<tr>
<th>Donor lysate</th>
<th>Chl(^{-})/Mut(^{+})</th>
<th>Recipient RK1009</th>
<th>Recipient W3350</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chl(^{-})/Met(^{+})</td>
<td>Per cent co-transduction</td>
<td>Chl(^{-})/Trp(^{+})</td>
</tr>
<tr>
<td>3</td>
<td>0/150</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>0/150</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>0/150</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>0/150</td>
<td>24/73</td>
<td>33</td>
</tr>
<tr>
<td>20</td>
<td>0/150</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>34</td>
<td>0/150</td>
<td>0/100</td>
<td>0</td>
</tr>
<tr>
<td>36</td>
<td>0/150</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Broth</td>
<td>0/0</td>
<td>0/40</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^{a}\) Plkc lysates were made from each of the mutants and used to infect two recipient strains. The selected markers were MetE\(^{+}\), Trp\(^{+}\) and Bio\(^{+}\). Chlorate resistance is the unselected marker in each case. Broth was also added to each recipient in place of phage, before plating on each of the selective media, to detect the frequency of revertants. Revertants occurred only when selecting for trp. The final cotransduction frequency was corrected for the number of revertants.

**Table 3. Comparison of nitrate reductase, succinic and formic dehydrogenases, and cytochrome *b* in mutants and wild type**

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Nitrate reductase*</th>
<th>Cytochrome <em>b</em></th>
<th>Succinic* dehydrogenase</th>
<th>Formic* dehydrogenase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\text{mg} \cdot \text{min}^{-1} \cdot \text{mg}^{-1})</td>
<td>(\text{mg} \cdot \text{min}^{-1} \cdot \text{mg}^{-1})</td>
<td>(\text{mg} \cdot \text{min}^{-1} \cdot \text{mg}^{-1})</td>
<td>(\text{mg} \cdot \text{min}^{-1} \cdot \text{mg}^{-1})</td>
</tr>
<tr>
<td>Wild-type</td>
<td>0.318</td>
<td>2.1</td>
<td>3.2</td>
<td>12.4</td>
</tr>
<tr>
<td>34 (chlD)</td>
<td>0.123</td>
<td>0.034</td>
<td>1.9</td>
<td>3.7</td>
</tr>
<tr>
<td>19 (chlC)</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>3.2</td>
<td>7.5</td>
</tr>
<tr>
<td>36chlB</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>3.1</td>
<td>6.3</td>
</tr>
<tr>
<td>16 (chlA)</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>1.9</td>
<td>2.4</td>
</tr>
<tr>
<td>20 (chlA)</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>4.5</td>
<td>3.7</td>
</tr>
<tr>
<td>3 (chlE)</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>2.4</td>
<td>2.1</td>
</tr>
<tr>
<td>13 (chlE)</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>2.4</td>
<td>1.9</td>
</tr>
</tbody>
</table>

\(^{a}\) Nitrate reductase and cytochrome measurements were made on Triton-soluble extracts of the envelope fraction of cells grown anaerobically to late log phase in minimal salts containing 0.5% Casamino Acids, 0.5% glucose, and, where specified, 0.1% nitrate. To express the amount of cytochrome *b* present, the difference between the peak at 558 nm and the trough at 578 nm (Fig. 1) was measured in optical density units and divided by micrograms of protein per milliliter.

\(^{a}\) Succinic and formic dehydrogenases were measured on particulate fractions of cells grown aerobically to late log phase in minimal salts plus 1% Casamino Acids. Enzyme activities are expressed in micromoles of substrate converted per minute per milligram of protein.

TABLE 2. Transduction mapping with phage Plke*
that of the wild type grown with nitrate. The addition of nitrate to the growth medium caused the production of increased amounts of cytochrome b in the mutants of the types chlA, chlB, chlC, and chlD, but not in chlE mutants.

Typical cytochrome scans on Triton extracts of the membranes of the parent and one mutant (13, chlE) are shown in Fig. 1. To determine whether mutation to chlorate resistance causes a general loss of cytochrome b-linked membrane-bound enzymes, succinic dehydrogenase levels were measured in cultures grown aerobically on minimal salts with Casamino Acids. The mutants all had succinic dehydrogenase activities similar to that found in the wild type. Formic dehydrogenase was also measured in all the mutants. In cultures growing aerobically in minimal salts with Casamino Acids, formic dehydrogenase activity was detectable only in the wild type and the chlC mutant. Under these growth conditions, formic dehydrogenase activity in chlC was only about 1% that found in the wild type. When cultures were grown aerobically on minimal medium plus glucose, causing the formic dehydrogenase activity of the wild type to be lowered, the amount of enzyme measured in the chlC mutant was 60% that in the wild type. When the combined reactions of formic dehydrogenase and hydrogenase (formic hydrogenlyase) were measured by the amount of gas trapped in a fermentation tube, only the chlC mutant produced any gas, and its formation occurred at a rate of about 60% that of the wild type. This crude method of hydrogenlyase measurement appears to correlate perfectly with H2 production from formate measured in a Warburg apparatus (10).

Ruiz-Herrera and DeMoss (12) also report inability to measure phenazine methosulfate-linked formic dehydrogenase in cultures which produced significant amounts of gas.

**Examination of membrane proteins by polyacrylamide gel electrophoresis.** One explanation for the pleiotropic effects of the chlorate-resistant mutations is that they are caused by a change in organization components, affecting the structure of the cell membrane (4). To find changes in the proteins of the membrane, the cytoplasmic membranes from each of the mutants and the wild type were separated from the cell wall protein by Triton extraction, and these solubilized proteins were examined by polyacrylamide gel electrophoresis. Figure 2 shows that the addition of nitrate to wild-type *E. coli* growing anaerobically in complex medium caused increased formation of three of the membrane proteins. When nitrate was omitted, proteins 1 and 2 were substantially decreased and protein 4 was not detectable. The chlB mutant, grown under the same conditions in the presence of nitrate, had a gel pattern which was essentially the same as that of the nitrate-grown wild type, with no changes in the proteins present in the membrane. Mutants of the type chlC, chlD, and chlE grown in the presence of nitrate all gave similar gel patterns; proteins 1 and 2 were completely undetectable. A fourth pattern was given by the chlA mutants. In this case, proteins 3 and 4 were undetectable. Individual gels of each of these membrane protein preparations were compared visually as well as by their optical density scans. Split gels (15) containing both the wild type and mutant on the same gel were also prepared so that a more exact comparison could be made.
Identification of one of the missing membrane components. In an attempt to ascertain the nature of the proteins missing from the membranes of the mutants, the enzyme nitrate reductase was purified by diethylaminoethyl column chromatography (Figure 3) and compared to the other wild-type proteins on polyacrylamide gels. Since the chlC gene has been reported to be the structural gene coding for nitrate reductase (8), this enzyme protein should be more likely to be missing from the membrane proteins of the chlC mutant than from any other mutant. When the wild type and the chlC mutant were grown aerobically on minimal medium with sodium succinate and KNO₃, large amounts of nitrate reductase were made by the wild type. Enzyme production appeared to be equal to the amount of nitrate reductase that could be measured in anaerobic broth cultures supplemented with nitrate. No nitrate reductase was detectable in the mutant. When the membrane proteins of mutant and wild type were compared on polyacrylamide gels (Fig. 4), a major band, present in the wild type, was missing in the mutant. This same band was the only one present when the purified nitrate reductase was examined on polyacrylamide gels. On split gels, the purified enzyme band corresponded exactly to protein 1 (Fig. 2) of the wild-type proteins.

DISCUSSION

Nitrate reductase, formic dehydrogenase, and cytochrome b are all affected by the mutation to chlorate resistance. Although nitrate reductase and formic dehydrogenase activities (except in chlC mutants) become severely reduced or undetectable, cytochrome b levels are only partially reduced. This tends to support the findings of Ruiz-Herrera and DeMoss (12) that there may be two distinct forms of cytochrome b, only one of which is involved in anaerobic electron transport to nitrate. At least two of these components, nitrate reductase and cytochrome b, are increased by the addition of nitrate to the growth medium, and evidence (10) has been presented that formic dehydrogenase levels are also ele-
Purification of nitrate reductase on diethylaminoethyl cellulose. Upper solid line shows the NaCl gradient, lower solid line shows protein (as measured by $^3$H counts), and dashed line shows nitrate reductase activity. RK20 was grown to late log phase on minimal medium containing 1% succinate, 0.1% KNO$_3$, and 500 µCi each of $^3$H-leucine and $^3$H-tyrosine per liter. Triton-soluble extracts of the envelope fraction were prepared as in Materials and Methods, except that the final Triton extraction was made in 0.01 m phosphate buffer (pH 7.8). The diethylaminoethyl column (24.5 by 1.5 cm) was equilibrated with the same buffer plus 1% Triton and $10^{-4}$ m mercauptoethanol (all final concentrations). After adding the Triton extract containing 20 mg of protein, a 320-ml gradient from 0 to 0.2 m NaCl in the same buffer was added, followed by 40 ml of 0.2 m NaCl in the same buffer. Fractions 129 to 136 were pooled. Purification of these fractions over the starting Triton-soluble material was 21-fold, and 100% of the enzyme activity was recovered. Seventy-five percent of the protein applied to the column was recovered.

vated by the presence of nitrate. These three components have been proposed (17) to be associated in the pathway for electron transport to nitrate:

$$\text{Formate} \rightarrow \text{formic dehydrogenase} \rightarrow \text{cytochrome b} \rightarrow \text{nitrate reductase} \rightarrow \text{nitrate}$$

Succinic dehydrogenase is also a membrane-bound, cytochrome b-linked enzyme (7); however, it is found predominantly in aerobic cultures, and succinate serves as an extremely poor donor of electrons to nitrate (6). When this enzyme was measured in the chlorate-resistant mutants, its activity was similar to that found in the wild type, in each case. The fact that all mutants grew as well as the wild type with succinate used as the sole carbon source is further evidence that the level of this enzyme is normal in the mutants. It has been reported that mutants with reduced amounts of cytochrome $b_{55}$ under anaerobic conditions produce normal cytochrome $b$ components under aerobic conditions (12). Thus, the pleiotropic effect of these mutations is

Fig. 3. Absorbance scans of polyacrylamide gels. Below each scan is a representation of the gel as in Fig. 2. Wild type and the chIC mutant were grown and prepared as in Fig. 3, freed of lipid and Triton, and separated on 7.5% gels. Nitrate reductase was purified from the wild type as in Fig. 3, and the purified nitrate reductase fractions from the diethylaminoethyl column were pooled and dialyzed overnight in the column buffer to remove the salt. This material was concentrated to half of its starting volume in an Amicon ultrafiltration cell and treated as all other preparations for electrophoresis.
not due to a general loss of cytochrome b-linked enzymes but appears to be confined to the components associated in electron transport to nitrate. This indicates that there might be a small particulate complex, located on a specific portion of the membrane, which contains the components of the system. This is further evidenced by the finding of Azoulay and Puig (3) that a particulate fraction, containing reconstituted nitrate reductase activity and cytochrome b, was obtained after incubation of a mixture of soluble extracts from two mutants with different genetic loci. The gel patterns indicate that there are at least four proteins in the cytoplasmic membrane which are involved in the functioning of the nitrate system. Only one of these has been identified and is the enzyme nitrate reductase. In nitrate-induced cells, this is a major component of the cytoplasmic membrane. This same enzyme is present in anaerobically or aerobically grown nitrate-induced cells. In mutants chlC, chlD, and chlE, the band corresponding to this enzyme is not found in gels of the membrane. It is not known whether the enzyme is present in the soluble fraction. Of particular interest are the mutants chlA and chlB, in which the nitrate reductase band is present in the cytoplasmic membrane but, for unknown reasons, does not function. This might be due to incorrect placement of the enzyme onto the membrane or to the absence of another component of the system, although the benzyl viologen-linked reaction used to assay the enzyme should not require participation by other components (e.g., cytochrome b). There are many possibilities in regard to the nature of the other proteins missing in the membranes of the various mutants. They could be involved in the transport of nitrate, molybdate, or selenite (10), or one might be a cytochrome protein. It is also possible that one or more of these proteins could be the organizational protein which holds all the components of the nitrate system in their proper location on the cytoplasmic membrane. Since such organizational proteins have not been shown to exist in E. coli, the chlorate system offers a good genetic and biochemical tool for investigating the existence of such proteins.

ACKNOWLEDGMENTS

This investigation was supported by National Science Foundation grant GB-8142, by Public Health Service Research Career Development Award GM22053 to C.A.S., and by National Institutes of Health postdoctoral fellowship GM45542 awarded to C.H.M.

LITERATURE CITED