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Subcloning of the Cloacin DF13/Aerobactin Receptor Protein and Identification of a pColV-K30-Determined Polypeptide Involved in Ferric-Aerobactin Uptake

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A plasmid containing a pColV-K30 fragment that encoded only for the cloacin DF13/aerobactin receptor protein was constructed. *Escherichia coli* cells harboring this plasmid were sensitive to cloacin DF13 but were unable to take up ferric-aerobactin. Another pColV-K30-determined polypeptide (molecular weight, 50,000), localized in the membrane fraction, was essential for the uptake of ferric-aerobactin.

In *Escherichia coli*(ColV-K30), the genes involved in aerobactin-mediated uptake of iron and cloacin DF13 sensitivity are located on the ColV-K30 plasmid (2, 10, 11). The cloning of a 6.5-kilobase (kb) fragment of pColV-K30 into pBR322 was recently described (6). *E. coli* cells harboring this recombinant plasmid, designated pFS8, are sensitive to cloacin DF13 and capable of ferric-aerobactin uptake but are unable to use the iron for growth. The plasmid encodes for two pColV-K30-determined polypeptides with molecular weights of 74,000 and 50,000. The 74,000-dalton polypeptide is the outer membrane receptor protein for aerobactin and cloacin DF13 (1, 4, 6), but the function of the 50,000-dalton polypeptide is not known. *E. coli* cells harboring a deletion derivative of pFS8, designated pBO310, that encodes only for the 50,000-dalton polypeptide are not sensitive to cloacin DF13 and are not capable of taking up ferric-aerobactin (6). In this report we describe the construction of another deletion derivative of pFS8 that encodes for the cloacin DF13/aerobactin receptor protein only. We present evidence that this receptor protein is the only plasmid-specified gene product which is essential for cloacin DF13 sensitivity. Uptake of ferric-aerobactin, however, requires the expression of both the receptor protein and the 50,000-dalton polypeptide. The subcellular localization of this 50,000-dalton polypeptide is also presented.

The physical map of pFS8 and the positions of the cleavage sites for various restriction enzymes are shown in Fig. 1. The gene for the 50,000-dalton polypeptide is located on the 3.1-kb large *Eco*RI-*Bam*HI fragment (6). This suggested that the gene for the cloacin DF13/aerobactin receptor protein might be located on the 3.4-kb large *Eco*RI-*Bam*HI fragment to the left of the unique *Eco*RI site in the cloned pColV-K30 fragment. Therefore, the 3.8-kb large *Eco*RI fragment was subcloned into the vector pACYC184. None of the recombinant plasmids, however, expressed the 74,000-dalton receptor protein. This indicated that the unique *Eco*RI site of the pColv-K30 fragment might be located in the gene encoding the receptor protein. Since there were no other suitable restriction sites known that could be used to construct the desired clone by complete digestion (Fig. 1), a partial digest of pFS8 with *Bgl*II was carried out. The partial digest was separated on an agarose gel (6), and a DNA band of about 9.5 kb was cut out. This DNA fragment was lacking the 1.0-kb large *Bgl*II fragment to the right of the unique *Eco*RI site (Fig. 1). The DNA was eluted from the agarose gel, recircularized, and used to transform competent cells of minicell-producing *E. coli* DS410 (6). Transformed cells were selected for resistance to ampicillin and sensitivity to cloacin DF13 (6). Twelve cloacin DF13-sensitive colonies were isolated an analyzed for their plasmid content. They all contained a 9.5-kb large plasmid which, upon digestion with restriction enzymes, was identified as pFS8, lacking the 1.0-kb large *Bgl*II fragment. One of these clones, designated pLO1, was used for further characterization (Fig. 1).

To determine which polypeptides were encoded by pLO1, minicells were isolated from *E. coli* DS410 harboring pLO1, labeled with [^{35}S] methionine, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig.

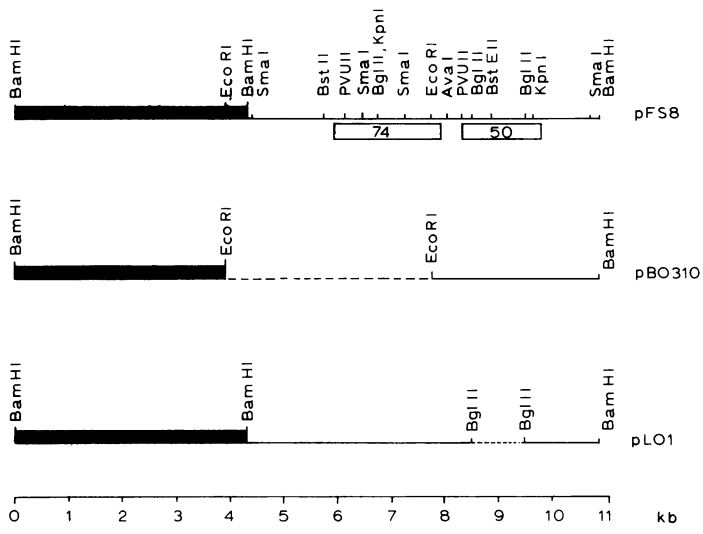

FIG. 1. Physical maps of pFS8, pBO310, and pLO1. The heavy lines represent the vector pBR322; the thin lines represent the inserted pColV-K30 DNA fragments. Cleavage sites for the various restriction enzymes are indicated. The putative location of the genes encoding the 74,000 (74)- and 50,000 (50)-dalton polypeptides are indicated in their approximate positions.

2). In addition to the polypeptides encoded by pBR322, only the 74,000-dalton receptor protein was detected. This indicated that the 1.0-kb deletion in pLO1 effectively eliminated the 50,000-dalton polypeptide. No additional polypeptide bands of lower mass were observed.

E. coli K-12 F176 *thr leu lacY tonA* Rif^r (6) was used to determine the number of outer membrane receptor proteins expressed by pLO1, pFS8, and pColV-K30. Therefore, pLO1 and pFS8 were transferred to *E. coli* F176 by transformation. Plasmid pColV-K30 was transferred by conjugation as described previously (6). The number of receptor molecules was determined after growth in Lab-Lemco broth (Oxoid Ltd., London, England) (6). Cells harboring pLO1 possessed about twice as many receptor molecules (9,800) as cells harboring pFS8 (5,100) and seven times as many as cells harboring pColV-K30 (1,400). Iron deprivation had no effect on the number of receptor molecules of cells harboring pLO1 or pFS8. The sensitivity of cells harboring pLO1, pFS8, or pColV-K30 to cloacin DF13 was determined on plates sprayed with different dilutions of a solution of cloacin DF13 (6). Cells harboring pLO1 were almost as sensitive to cloacin DF13 as cells harboring pFS8 or the intact pColV-K30 plasmid. These results indicated that the 50,000-dalton polypeptide is not involved in cloacin DF13 sensitivity and that there is no linear relationship between the number of receptor molecules per cell and the sensitivity of cells harboring pLO1 as compared with that of cells harboring pFS8 might be caused by an excessive amount of functionally inactive receptor proteins (5).

Cells of strain F176 harboring pLO1 could be protected against the lethal action of cloacin DF13 by aerobactin. About 10 pmol of aerobactin per ml reduced the activity of 1.5 pmol of cloacin DF13 per ml to 50%. This indicated that the receptor protein encoded by pLO1 was still capable of binding aerobactin (10).

To investigate whether the 50,000-dalton polypeptide is involved in aerobactin-mediated uptake of iron, we transformed *E. coli* UT4400 *entA cbr a*, defective in the biosynthesis of enterochelin (7), with pLO1 and pFS8 and used it for ^{55}Fe uptake studies (Fig. 3). *E. coli* UT4400 cells harboring pLO1 were not capable of taking up iron if complexed with aerobactin in contrast to cells harboring pFS8. This indicated that the 50,000-dalton polypeptide is involved in the uptake or transport of ferric-aerobactin across the outer membrane of *E. coli* cells. The raised level of iron found associated with cells harboring pLO1 can be explained by absorption of labeled ferric-aerobactin to the receptor molecules at the surfaces of the cells.

To demonstrate that the 50,000-dalton polypeptide is involved in the uptake of ferric-aerobactin, we carried out a complementation experiment. The pColV-K30-derived DNA fragment of pLO1 was isolated and inserted into the single *Bam*HI site of pACYC184. The resulting recombinant plasmid, designated pWK40, was isolated and used to transform competent

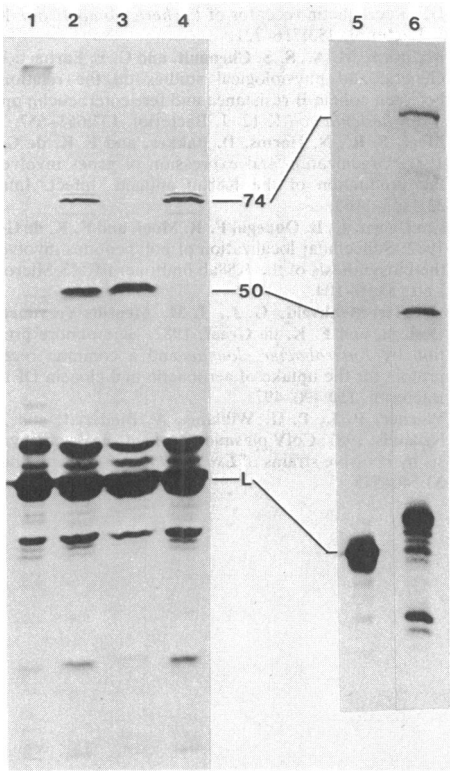

FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of minicells harboring pBR322 (lane 1), pFS8 (lane 2), pBO310 (lane 3), and pLO1 (lane 4) and of minicells fractionated into a soluble fraction (lane 5) and a membrane fraction (lane 6). Minicells were isolated and labeled with [^{35}S] methionine as described previously (8). The labeled polypeptides were analyzed on 14% sodium dodecyl sulfate-polyacrylamide gels (6). Furthermore, minicells harboring pFS8 were separated into a soluble fraction and a membrane fraction by passing them twice through a French pressure cell. After removal of unbroken cells at 5,000 × *g*, membranes were collected by centrifugation at 200,000 × *g* for 2 h and solubilized in sample buffer. The supernatant was lyophilized and also solubilized in sample buffer. Masses are indicated in kilodaltons. β-lactamase (L) was used as an indicator for the soluble fraction (9).

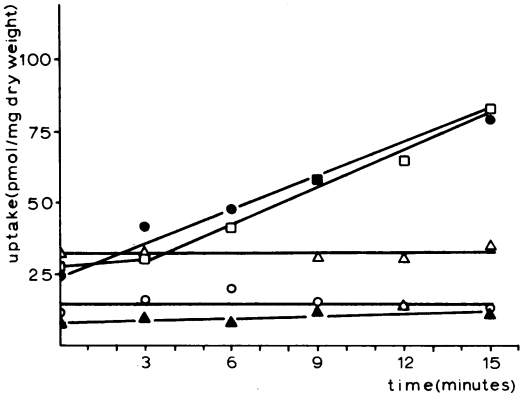

FIG. 3. Uptake of ferric-aerobactin by *E. coli* UT4400 (○) and by *E. coli* UT4400 containing pLO1 (△), pFS8 (□), pBO310 (▲), or pBO310 and pWK40 (●). The preparation of iron-deprived cultures as well as the uptake studies were performed as described previously (6). Ferric-aerobactin was administered at a concentration of 1 μM.

cells of *E. coli* UT4400, already harboring pBO310. A chloramphenicol- and ampicillin-resistant and cloacin DF13-sensitive colony was isolated and used for ferric-aerobactin uptake studies (Fig. 3). Strain UT4400 containing only pBO310 or pWK40 did not take up ferric-aerobactin. When both pBO310 and pWK40 were present, the uptake of ferric-aerobactin was restored to the same level as that found in strain UT4400 cells harboring pFS8. This showed that the 50,000-dalton polypeptide is involved in ferric-aerobactin uptake or transport or both. The lack of ferric-aerobactin uptake in cells harboring pLO1 cannot be due to an incomplete receptor, since the deletion in pLO1 can be complemented in *trans*. Furthermore, cloacin DF13 and ferric-aerobactin are able to compete for the same receptor sites.

The subcellular localization of the 50,000-dalton polypeptide was carried out in *E. coli* DS410 minicells harboring pFS8 (Fig. 2). The 74,000- and 50,000-dalton polypeptides were both found in the membrane fraction. Only a minor amount of the 50,000-dalton polypeptide was found in the soluble protein fraction that contained virtually all of the β-lactamase. This suggested that the 50,000-dalton polypeptide either is located in or at the inner membrane or is loosely associated with the outer membrane. In a previous study (6) it was found that the 50,000-dalton polypeptide is not synthesized with a leader sequence, a common feature for periplasmic or outer membrane proteins. Therefore, this polypeptide is most likely located in or at the cytoplasmic membrane.

Since uptake of cloacin DF13 does not require the 50,000-dalton polypeptide, cloacin DF13 probably enters the cell by a different route. Whether chromosome-determined products are required for cloacin DF13 sensitivity remains to be investigated. Preliminary experiments showed that one of the outer membrane pore proteins might be needed for cloacin DF13 sensitivity. Similar results have been published for colicin A (3).

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