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Genetic Analysis of Sequences in Maltoporin That Contribute to Binding Domains and Pore Structure

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Maltoporin (LamB protein) is a maltodextrin transport protein in the outer membrane of *Escherichia coli* with binding sites for bacteriophage λ and maltosaccharides. Binding of starch by bacteria was found to inhibit swarming of *Escherichia coli* in soft agar plates; the inhibition was dependent on the maltodextrin affinity of maltoporin. On the basis of this observation, chemotactic cell-sorting techniques were developed for the isolation and analysis of mutants with an altered starch-binding phenotype. Fifteen *lamB* mutations generated by hydroxylamine and linker mutagenesis, as well as spontaneous mutations, were analyzed. The effects of the mutations on starch and lambda-binding, as well as transport specificity, were assayed. Mutations that affect residues near 8 to 18, 74 to 82, and 118 to 121 were found to affect starch binding and maltodextrin-selective functions strongly, confirming and extending previous results with substitutions at these regions. Substitutions and insertions in two previously undefined regions in the protein, in or near residues 194 and 360, also resulted in defects in maltodextrin-specific functions and indicate that C-terminal parts of the protein also contribute to the discontinuous binding and pore domains. There was a detectable transport defect in all binding-affected mutants, and one mutation caused near-total pore blocking towards both maltose and nonmaltoside. The highly discontinuous phage λ-binding site was affected by mutations near residues 9 and 10 and 194, as well as previously established regions near residues 18, 148 to 165, 245 to 259, and 380 to 400. The significance of these mutations is discussed in the context of a model of the functional topology of maltoporin. The additional role of regions near residues 10 and 120 in maltoporin assembly, as well as starch binding, was suggested by the temperature-sensitive biogenesis of maltoporin in strains with one- or two-codon insertions at these sites.

Maltoporin (LamB protein) is a component of the maltose- and maltodextrin-specific transport system in *Escherichia coli* (32) and is encoded by the *lamB* gene (6). Its physiological role is to permit access of dextrins above maltotriose across the outer membrane (34); maltoporin is also the bacteriophage lambda receptor protein (29). On the basis of reconstitution studies with purified protein, maltoporin has been shown to form channels highly selective, specifically for maltooligosaccharides, among larger sugars (3, 20, 24–26).

The presumed basis of the selectivity of maltoporin, as with other transport proteins, is a stereospecific binding site for the substrate. A maltodextrin-binding site has indeed been demonstrated (14), and its affinity correlates well with transport affinities measured in vivo and in vitro (3, 13, 19). The unique feature of this binding site among transport proteins is that it is readily amenable to genetic analysis, and several residues that influence binding and transport have been recently identified (18; A. Charbit, K. Gehring, H. Nikaido, T. Ferenci, and M. Hofnung, J. Mol. Biol., in press). Such a genetic approach should lead to identification of the residues that contribute to the selectivity filter in maltoporin channels. In this study, we report on the binding and transport properties of mutants with further substitutions and insertions that affect the maltodextrin recognition site.

Previously isolated mutants altered in maltodextrin binding were enriched through affinity-chromatographic cell sorting, on the basis of accessibility of the maltodextrin-binding site in the intact cell to macromolecular, immobilized starch (11). The genetic analysis of the maltodextrin-binding site using affinity-chromatographic approaches was hampered by the limitation that each *lamB* mutant had to be isolated through repeated chromatographic enrichment cycles of bacterial populations (up to 27 in the case of Clune et al. [7]). Also, the binding phenotype of each *lamB* recombinant or clone had to be previously tested in individual column chromatographic experiments. These rate-limiting steps stimulated development of the novel plate assay for starch binding described in this paper. This new technique permits rapid isolation and screening of mutants specifically altered in maltoporin.

Studies of maltoporin variants with amino acid substitutions that influence the binding of phage lambda (16), surface-accessible monoclonal antibodies (9), and starch (18) is beginning to permit the footprinting of these macromolecular binding sites on the surface of the protein (Charbit et al., in press). This extensive mutant information has also been incorporated into a model of maltoporin folding across the outer membrane (Charbit et al., in press). The properties of the new substitutions and insertions described in this paper provide additional evidence in support of the latest model. The results presented confirm the importance of regions near residues 10, 80, and 120 in starch binding and extend the footprint of the starch-binding site to residues near 194 and 360 in the mature sequence. The definition of the phage lambda-binding site has also been extended to incorporate the 9-to-10 and 194 regions as well.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. Luria-Bertani

TABLE 1. Bacterial strains

Strain	Genotype	Source or reference
HfrG6	HfrG6 *his*	17
pop6510	F⁻ *thr leu metA lacY tonA supE recA56 srl*::Tn*10 lamB* (*dex5*)	4
BW1022	HfrG6 *metA trpE aroB*	11
BW2508	HfrG6 *his malBΔ10*	18
BW2514	HfrG6 *his lamB1330*	This study
BW2518	HfrG6 *his lamB1040*	This study
BW2520	HfrG6 *his* Δ(*malK'-lamB*)*111*	This study
BW2524	HfrG6 *his lamB1400*	This study
BW2525	HfrG6 *his lamB1500*	This study
BW2605	HfrG6 *trpE aroB* Δ(*malK'-lamB*)*111*	This study
BW2634	HfrG6 *trpE aroB*	This study
BW2636	HfrG6 *trpE aroB lamB203*	This study
BW2637	HfrG6 *trpE ompR*::Tn*10*	This study
BW2638	HfrG6 *trpE ompR*::Tn*10 lamB1002*	This study
BW2639	HfrG6 *trpE ompR*::Tn*10 lamB203*	This study
BW2640	HfrG6 *trpE ompR*::Tn*10 lamB1004*	This study
BW2641	HfrG6 *trpE ompR*::Tn*10 lamB146*	This study
BW2642	HfrG6 *trpE ompR*::Tn*10 lamB153*	This study
BW2644	HfrG6 *trpE aroB lamB375*	This study
BW2645	HfrG6 *trpE aroB ompR*::Tn*10 lamB147*	This study
BW2646	HfrG6 *trpE ompR*::Tn*10 lamB375*	This study

(LB) medium was made as described by Davis et al. (8); minimal medium A was prepared as described by Miller (23). Chemotaxis minimal media were based on that of Parkinson (28) and contained 0.25% agar. The attractant was 0.1 mM ribose, and the starch concentration in the plates was 2 mg/ml. Ampicillin was present at 100 μg/ml in LB and 50 μg/ml in minimal media.

General genetic techniques. Standard genetic manipulations were done as described by Miller (23) and Maniatis et al. (22). Cloning of chromosomal *lamB* mutations was exactly as described by Heine et al. (18) and resulted in *lamB* in a plasmid configuration equivalent to pHCP2 (6). Subcloning for mapping and sequencing of mutations was performed by exchanging appropriate *lamB*-containing DNA restriction fragments extracted from low-melting-temperature agarose gels (18).

Construction of pAM117 and isolation of mutations. Plasmid pAM117 (Fig. 1) was constructed from pHCP2 (6) by replacing the *malK*-internal *Eco*RI-*Sal*I fragment with the *Eco*RI-*Sal*I fragment of the multicloning site from pUC18 (35). The *lamB*-distal *Sac*I-*Pvu*II fragment was also deleted from pHCP2. The unique *Bam*HI site of the multicloning site was inactivated (by *Bam*HI digestion and filling in of overhangs) to facilitate mapping of *Bam*HI linker insertions (see below).

For linker mutagenesis, pAM117 was digested with *Hpa*II in the presence of 100 μg of ethidium bromide per ml under conditions which gave complete digestion in the absence of ethidium bromide; the presence of ethidium bromide resulted in the appearance of about 25% of the total DNA as linear, plasmid-size DNA (27). Full-size linear plasmid DNA was cut out from a 0.7% low-melting-temperature agarose gel and extracted at 65°C after dilution to less than 0.15% agarose. The extracted DNA was treated sequentially with phenol, phenol-chloroform, and chloroform and then ethanol precipitated. A 650-ng sample of the linear fragment was ligated as described by Barany (2), with 0.1 optical density unit (at 260 nm) of two-amino-acid Barany (TAB) linker pCGGATC (Pharmacia, Sydney, Australia) in a 15-μl total volume for 1 h at 12°C. The ligation mixture was transformed into BW2508, and ampicillin-resistant transformants were selected on LB plates. The plasmids were analyzed for the presence of a *Bam*HI site and trimmed of excess linker, and the insertions were mapped in double digests with *Bam*HI and enzymes with known restriction sites.

Plasmid DNA was chemically mutagenized with hydroxylamine by using a modification of the procedure described by Eichenlaub (10). A mutagenesis mixture containing 100 μl of pAM117 (200 μg of DNA per ml) and 400 μl of freshly prepared 1 M NH_2OH, pH 6.0, was incubated at 55°C for 45 min. The mutagenized DNA was ethanol precipitated, washed with 70% ethanol to remove any remaining NH_2OH, and then suspended in 50 μl of TE buffer (22), pH 8.0. For mutant selection, 5 μl of mutagenized pAM117 was transformed into 200 μl of competent BW2508. Phenotypic expression of the β-lactamase gene was allowed for 40 min at 37°C before the bacteria were plated onto LB plates containing ampicillin (100 μg/ml). The >1,000 ampicillin-resistant transformants obtained on each plate were collected into separate pools and diluted into fresh LB-ampicillin medium. Seven independent pools of transformants were subjected to selection on ribose-starch chemotaxis plates as described below.

Mutations derived in plasmid-borne *lamB* were recombined into the chromosome to obtain stable expression for transport comparisons. Mutated plasmids in a pAM117 configuration were converted into a pHCP2 configuration, containing approximately 1 kilobase of *malK* upstream of *lamB*. Each of these plasmids was transformed into BW2605 (a strain with a chromosomal *lamB* deletion extending into *malK*), and Mal⁺ recombinants were selected by plating on maltose minimal plates. P1 lysates of the recombinants were prepared and used to transduce the mutant *lamB* alleles into

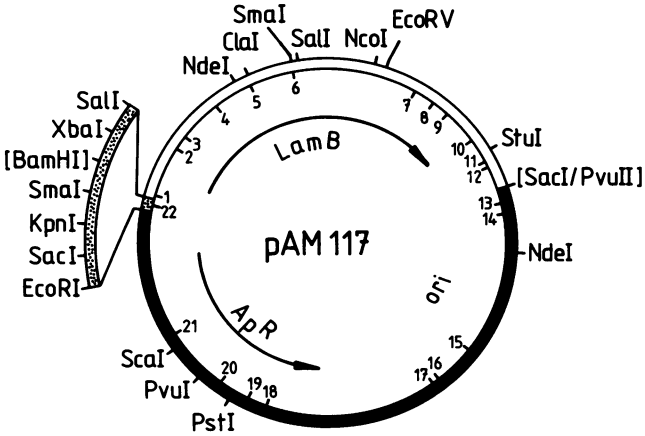

FIG. 1. Construction of, and *Hpa*II sites in, plasmid pAM117. pAM117 is 3,990 base pairs long and was derived from plasmid pHCP2 (6). pAM117 contains the whole of the *lamB* gene downstream of the *Sal*I site in pHCP2, but the *malK*-internal *Eco*RI-*Sal*I fragment in pHCP2 has been replaced by the *Eco*RI-*Sal*I fragment of the pUC18 multicloning site. The *lamB*-distal *Sac*I-*Pvu*II fragment in pHCP2 was also deleted to give the hybrid *Sac*I-*Pvu*II site in pAM117. The fusion of the vector at the *Pvu*II site led to deletion of the *rop* gene (1) and therefore to an increase in plasmid copy number (33). The light areas in the plasmid represent the remaining chromosomal *malB* insert in pAM117, the dark areas are from pBR322, and the dotted areas are from pUC18. The numbers inside the circle give the positions of the 22 *Hpa*II sites in pAM117 at which linker insertions were expected: eight of these (sites 2 to 9) were *lamB* internal.

BW2605 to ensure that only chromosomal *lamB* was transferred. Finally, to assist in pore studies, an *ompR*::Tn*10* mutation was introduced into the recombinant strains via P1 transduction, selecting tetracycline-resistant transductants. The lack of *ompF-ompC* porins in these strains was confirmed by analysis of outer membrane proteins in acrylamide gels (see below).

DNA sequencing. Mutations in *lamB* were mapped in pAM117-derived plasmids (i) to the *Sal*I-*Cla*I fragment, containing the early part of the gene, (ii) to the *Cla*I-*Nco*I fragment in the middle of *lamB*, or (iii) on the *Nco*I-*Stu*I fragment covering the end of the gene as previously described (18). The mutated DNA fragments were subcloned into M13mp18 or M13mp19 (35) and sequenced with the dideoxynucleotide method (30).

Selection and screening of dextrin-binding-negative mutants in chemotaxis plates. Mutant selection was performed in minimal chemotaxis agar plates containing 2 mg of starch per ml in which 0.25% microbiological agar was replaced by 0.1% high-gelling-temperature agarose plus 0.5% low-gelling-temperature agarose. These plates were semisolid when kept at 37°C. The plates were inoculated by stabbing into the agar 1 to 2 μl of concentrated cell suspension from the pooled mutagenized transformants (approximately 10^7 bacteria). After overnight incubation at 37°C, the agarose mixture was allowed to set at room temperature. A 0.2-ml volume of 20% glucose was added around the edges of the plate, and the plate was further incubated for 1 to 2 days at 37°C. After the agarose was set, the cells which swam away from the site of inoculation were fixed and formed colonies; these were picked and further analyzed after purification.

To screen mutants capable of swimming in the presence of starch, chemotaxis plates containing 0.24% microbiological agar and 2 mg of starch per ml were inoculated with purified colonies picked with a toothpick and incubated for 5 to 8 h at 37°C or overnight at 30°C. Ring formation was indicative of lack of starch binding.

Chromatographic starch-binding assay. Maltoporin-dependent binding of bacteria to starch was assayed on starch-Sepharose columns as previously described (11), with bacteria grown at 30°C.

Pore properties of maltoporin. The kinetics of maltose transport and its inhibition by maltohexaose were assayed in bacteria with chromosomal mutant *lamB* alleles but lacking other major porins (13). The non-maltodextrin-specific pore function in maltoporin was also assayed, with lactose as a substrate, as previously described (13). The bacteria were grown at 30°C to permit maltoporin expression at the permissive temperature for temperature-sensitive mutants.

Efficiency of plating of phage Lambda. The efficiency of plating of λ *vir* on strains of *E. coli* was assayed in strains grown at 30°C to permit expression of temperature-sensitive alleles. Strains were considered phage resistant when a 10^6-fold drop in plating efficiency was observed (5).

Maltoporin extraction and gel electrophoresis. The LamB expression of mutants was analyzed in sodium dodecyl sulfate-polyacrylamide gel electrophoresis of outer membrane proteins extracted by using two experimental approaches. The first extraction method, as previously described (12), was used to confirm the temperature sensitivity of mutants. The second, described below, was a more rapid extraction procedure and was used to screen mutants and recombinants. For the rapid extraction, overnight cultures grown in 5 ml of LB-ampicillin were harvested, washed in 50 mM Tris hydrochloride, pH 8, and suspended in 0.5 ml of 20% (wt/vol) sucrose–100 mM Tris hydrochloride, pH 8.

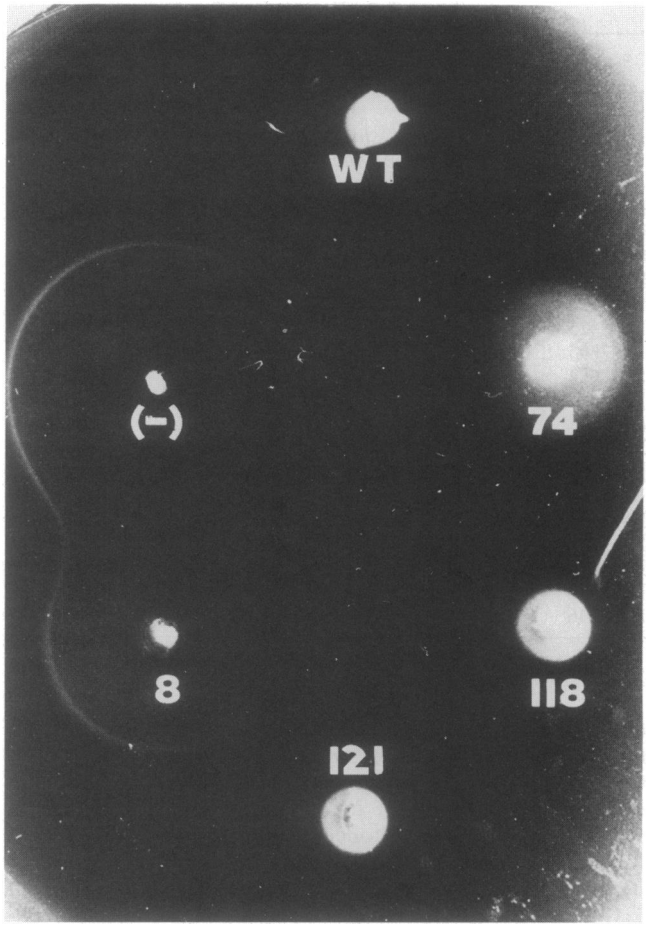

FIG. 2. Maltoporin affinity for starch and its influence on the swimming ability of *E. coli*. A ribose chemotaxis soft agar plate, containing 0.24% agar and 2 mg of starch per ml, was inoculated with strains that exhibit various alterations in maltoporin affinity for starch (18). The following strains were shown: HFrG6 (WT; wild type for *lamB*), BW2508 [(−); Δ*lamB*], BW2514 (74; *lamB1330* encoding a Trp74→Arg substitution), BW2524 (118; *lamB1400* encoding Tyr118→Phe), BW2525 (121; *lamB1500* encoding Asp121 →Ser), and BW2518 (8; *lamB1040* encoding Arg8→His). The plate was incubated overnight at 30°C and photographed.

Addition of 0.010 ml of 0.5 M EDTA and 0.020 ml of 10-mg/ml lysozyme and 15 min of incubation at 37°C led to the formation of spheroplasts. These were lysed upon addition of 0.5 ml of extraction buffer (2% Triton X-100, 10 mM MgCl, 50 mM Tris hydrochloride, pH 8)–0.050 ml of 1-mg/ml DNase plus RNase and further incubation at 30°C for 30 min. Membrane particles were sedimented in an Eppendorf centrifuge after 5 min of centrifugation, washed twice in 20% ethanol, and suspended in 30 μl of 4× concentrated gel sample buffer (21). Samples were boiled for 5 min before being loaded onto sodium dodecyl sulfate-polyacrylamide electrophoretic gels (21).

RESULTS

Isolation and analysis of starch-binding mutants in swarm plates. Starch was known to bind to the surface of *E. coli* in a maltoporin-dependent manner (14). The presence of starch in ribose chemotaxis soft agar plates (Fig. 2) was found to

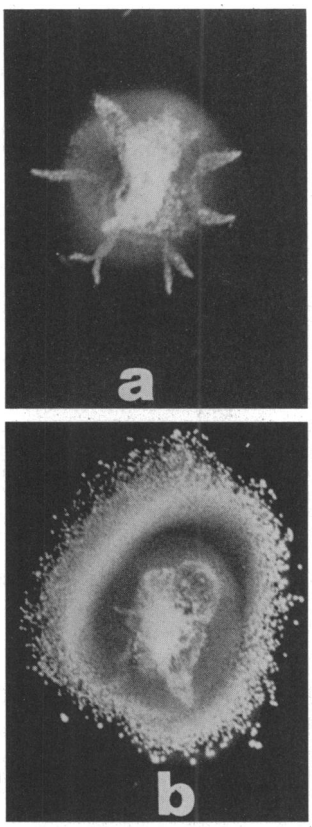

FIG. 3. Isolation of mutants which can swim in the presence of starch. Chemotaxis plates containing 2 mg of starch per ml–0.1% high-gelling-temperature agarose–0.5% low-gelling-temperature agarose were prepared and kept semisolid at 37°C. A plate was inoculated with wild-type strain HFrG6 by stabbing approximately 10^7 bacteria into the center of the plate. After overnight incubation at 37°C, the agarose mixture was allowed to set at room temperature; the site of inoculation is shown in panel A. After further overnight incubation with glucose, small colonies appeared, formed from cells which swam away from the site of inoculation (B).

prevent swimming of wild-type, motile strains of *E. coli* away from the site of inoculation. This inhibition of swimming was *lamB* dependent, since a strain with a *lamB*-null mutation showed ring formation characteristic of a normal chemotactic response (28). Furthermore, the size of the ring formed in ribose-starch plates was proportional to the inherent affinity of maltoporin in the outer membrane. A known *lamB* mutant (18) with a partial defect in starch binding (Trp74→Arg substitution) formed a diffuse ring larger than that of the wild type but smaller than that of a mutant with a strong starch-binding defect (Arg8→His) (Fig. 2). Maltoporin substitutions that lead to higher starch affinity (118 and 121 substitutions [18]) were also unable to swim in the presence of starch. These results demonstrated the sensitivity of this approach in the screening of binding site mutants.

Swimming inhibition by starch could also be used to select mutants with alterations in the maltodextrin-binding site. Bacteria in a population which could swim away from the site of inoculation in the presence of starch were specifically enriched for alterations in *lamB* function. Bacterial clones which swam away could be visualized in agar plates after the bacteria were frozen in place in the soft agar; this could be achieved by using low-gelling-temperature agar at 37°C for the swimming phase but dropping the temperature below 30°C to trap bacteria (Fig. 3). After addition of glucose (to supplement the low ribose concentration present as a carbon source in the plates), colonies distant from the site of inoculation could be grown as shown in Fig. 3. Of 22 isolated colonies picked from the plate in Fig. 3, 15 were phage λ resistant, and only 2 were wild type for starch binding. These results also indicated the specificity of the selection for maltoporin function. This procedure could be used to isolate spontaneous mutants altered in *lamB* encoded in either the chromosome or pAM117; selections that involved plasmids required plates containing ampicillin to reduce the numbers of isolates which gain *lamB* negativity through plasmid loss.

Selection and analysis of spontaneous and hydroxylamine-induced *lamB* mutations in plasmid pAM117. To facilitate linker mutagenesis (see below) and sequencing of mutations, the plasmid shown in Fig. 1 was constructed. Although the *lamB* gene in this plasmid has no controllable promoter, the maltoporin in the outer membrane expressed from this plasmid is approximately at 30% of the level of fully induced, wild-type, chromosomally encoded protein at 30°C and 50%

TABLE 2. Sequence changes in *lamB* mutants

Allele no.	Type of mutagenesis	Selection technique[a]	DNA change[b]	Amino acid change[c]
1001[d]	Hydroxylamine	Plate	197G→A	Arg8→His
1002[d]	Hydroxylamine	Plate	755G→A	Gly194→Asp
1004	Hydroxylamine	Plate	1252C→T	Arg360→Cys
1007	Hydroxylamine	Plate	1434G→A	Trp420→Stop
212	Spontaneous	Plate	197G→A	Arg8→His
210	Spontaneous	Column	197G→A	Arg8→His
203	Spontaneous	Plate	+TAC at 531–532	+Tyr at 119–120
375	Linker	TAB	+CGGATC at 200–201	+GlySer at 9–10
147	Linker	TAB	+CGGATC at 409–410	+AspPro at 79–80
153	Linker	TAB	+CGGATC at 721–722	+AspPro at 183–184
146	Linker	TAB	+CGGATC at 1299–1300	+AspPro at 361–362

[a] The *lamB* mutants carrying these mutations were selected either in starch-swarm plates or by column enrichment for starch binding-negative mutants (11). Linker insertions were isolated through the TAB procedure (2).
[b] The numbering of the nucleotide sequence is from the *SalI* site upstream of *lamB* (6).
[c] The numbering of the amino acid sequence is from the start of the mature maltoporin sequence.
[d] Three independent isolates with this mutation were sequenced.

TABLE 3. Phenotypic characterization of bacteria that express maltoporin variants

Amino acid change in LamB	Protein in outer membrane[a]	Lambda plating efficiency[b]	Starch binding (% retention)[c]	Growth on dextrins[d]	Transport affinity for[e]: Maltose (K_m, μM)	Transport affinity for[e]: Hexaose (K_i, μM)
Wild type	+	1	99	Dex^+	2.3	0.9
Arg8→His	+	1	56	Dex^+	4.5	23.3
+GlySer at 9–10	− (+ at 30°C)	10^{-9}	47	Dex^-	14.5	14.1
+AspPro at 79–80	+	1	88	Dex^+	7.4	2.5
+Tyr at 119–120	− (+ at 30°C)	1	42	Dex^-	NT	
+AspPro at 183–184	+	1	98	Dex^+	2.5	0.9
Gly194→Asp	+	10^{-6}	92	Dex^+	6.8	2.5
Arg360→Cys	+	1	44	Dex^+	10.6	3.5
+AspPro at 361–362	+	1	31	$Dex^\pm$	8.6	17.2
Trp420→Stop	−	0.3	17	Dex^+	ND	ND
LamB deletion	−	$<10^{-9}$	17	Dex^-	ND	ND

[a] Protein levels were compared as shown in Fig. 4. Symbols: +, wild-type levels; −, at least a fivefold reduction compared with the wild type.
[b] Plating efficiency with phage lambda was assayed in strains with *lamB* expressed in a pAM117 configuration in strain BW2508. Bacteria were grown at the permissive temperature (30°C).
[c] Starch binding was assayed with *lamB*'s in the chromosome, under full maltose induction. Bacteria were grown at 30°C.
[d] Growth on dextrins was assayed on MacConkey agar plates containing 0.4% maltooligosaccharide. The bacteria contained *lamB* in a pAM117 configuration in strain pop6510 at 30°C.
[e] Transport assays were carried out with chromosomal, fully induced *lamB*'s in an $OmpR^-$ background. NT, Not transported; ND, not determined.

of the wild type at 42°C. These values were estimated by densitometric scanning of acrylamide gels (results not shown) and were adequate for assays of binding phenotype but did not cause problems of overexpression.

Spontaneous mutations were selected by the plate technique described above; plasmids with mutations introduced by mutagenesis in vitro with hydroxylamine (10) were transformed into BW2508 (containing a deletion of chromosomal *lamB*) and clones with altered starch binding were enriched and identified by using the plate assay. Each of the mutations listed in Table 2 was from an independent isolate, from either different inocula (spontaneous mutations) or different batches of mutagenized plasmid. The mutations were mapped relative to particular restriction sites in *lamB* (*Cla*I, *Sal*I, and *Nco*I) by exchange of corresponding fragments between mutant and wild-type pAM117 and phenotype testing. The fragment containing the mutation was sequenced in two orientations cloned into M13, as previously described (18).

The most frequent mutation that gave a starch-binding negative phenotype was a CGT→CAT substitution resulting in Arg8→His (Table 2). This mutation has now been observed in seven independent spontaneous and hydroxylamine-induced *lamB* mutants, including two found in an earlier study (18). It is not clear why selections for binding negativity result in such a high frequency of mutations at this position. As shown earlier and in Table 3, this substitution affects neither the amount of maltoporin produced nor phage lambda binding.

The only other spontaneous mutation obtained that resulted in reduced starch binding was an insertion of a Tyr codon (TAC). This insertion occurred in the gene where two TAC codons encode Tyr residue 118 and 119; the mutation represents a duplication of one of these codons. The mutation results in a noticeable reduction in the amount of maltoporin present in the outer membrane at temperatures above 37°C but not at 30°C (Fig. 4). The protein, when present, permitted good binding of phage lambda, since the efficiency of plating of λ was hardly affected by the insertion (Table 3). Starch binding, in contrast, was strongly affected, even at the permissive temperature. This mutation also prevented maltodextrin permeation as detected by the Dex^-

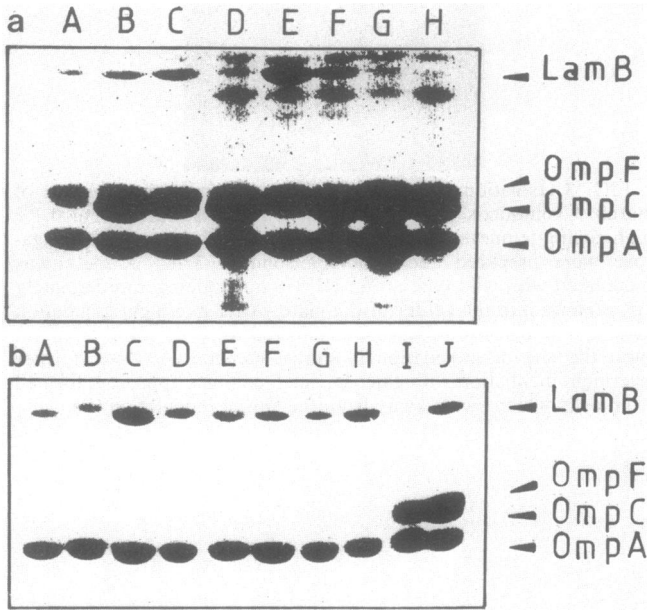

FIG. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis of outer membrane extracts of *lamB* mutants. For panel a, outer membrane extracts were prepared as previously described (12). Bacteria were grown at either 30°C (lanes A, C, E, and G) or 42°C (lanes B, D, F, and G). Strains containing chromosomally encoded *lamB* (lanes A to F) were grown on minimal medium plus 0.2% maltose. Plasmid-containing strains (lanes G and H) were grown on LB plus 50 μg of ampicillin per ml. Lanes: A and B, BW2634 (*lamB*$^+$); C and D, BW2636 (*lamB211*); E and F, BW2644 (*lamB375*); G and H, BW2508 containing pAM1007 (*lamB1007*). For panel b, outer membrane proteins extracted by the rapid procedure (as described in the text) were analyzed by electrophoresis in the presence of sodium dodecyl sulfate. The strains analyzed contained chromosomal, maltose-induced mutant *lamB*'s in an $OmpR^-$ background. Bacteria were grown at 30°C. The lanes contained the following: J, BW1022 (*lamB*$^+$ *ompR*$^+$); I, BW2605 (Δ*lamB ompR*$^+$); H, BW2637 (*lamB+ ompR*); G, BW2638 (*lamB1002 ompR*); F, BW2639 (*lamB203 ompR*); E, BW2640 (*lamB1004 ompR*); D, BW2641 (*lamB146 ompR*); C, BW2642 (*lamB153 ompR*); B, BW2645 (*lamB147 ompR*); A, BW2646 (*lamB375 ompR*).

phenotype and, as shown below, by the total absence of detectable pore activity in the mutant maltoporin.

Another mutation that resulted in reduced maltoporin levels in the outer membrane was due to conversion of the codon for the penultimate amino acid, Trp420, into a nonsense codon (TGA; Table 2 and Fig. 4). The lack of the last two residues of the native protein was sufficient to lead to perturbation of maltoporin assembly or stability, leading to at least a fivefold reduction in protein levels (Fig. 4). Again, as in the case of the TAC insertion, the residual protein formed could act as a good phage receptor (Table 3). The mutation also permitted dextrin fermentation and so did not abolish pore function, but the level of starch binding was reduced; this was probably due to the reduction in the number rather than the affinity of binding sites in this mutant.

Single amino acid substitutions at positions 194 and 360 both resulted in defects in starch binding, without a reduction in protein levels (Tables 2 and 3). The Arg360→Cys substitution did not affect phage lambda binding, but the Gly194→Asp substitution resulted in phage lambda resistance by the criterion of a 10^6-fold drop in plating efficiency (5). As with a number of other phage-resistant isolates previously investigated (Charbit et al., in press), the starch-binding defect that resulted from the 194 substitution was a partial reduction. Both of these substitutions are in parts of the gene where no previous point mutations were observed, even though the Gly194 substitution could have been selectable through phage resistance. The sequence change in *lamB1002* found by DNA sequencing suggested that the mutation generated a unique *Pvu*I site in the *lamB* gene; this was confirmed by restriction mapping.

Linker mutagenesis in pAM117. The observations that substitutions at residues 8, 74, 82, 118 to 121, and 360 in maltoporin resulted in a changed starch-binding phenotype (18 and above) stimulated the isolation of linker insertions in *lamB* near these coding regions. There were suitable *Hpa*II sites near coding regions for these residues so that the TAB linker mutagenesis approach of Barany (2) was adopted. Plasmid pAM117 was constructed so as to keep *lamB*-external *Hpa*II sites to a minimum (Fig. 1). Using the standard TAB mutagenesis procedure (2), two-codon insertions in *lamB* were isolated with additional amino acids inserted between residues 9 to 10, 79 to 80, 183 to 184, and 361 to 362; insertions outside *lamB* were also found (Table 2). Each of these insertions encodes a unique *Bam*HI site, which assisted in mapping of the insertions. There was a definite hot spot at position 79 to 80, and no insertions were found in potential sites near residues 26, 127, 336, and 381 in seven independent ligation-transformation experiments. Either these insertions cause a lethal phenotype or, more likely, cleavage with *Hpa*II in the presence of ethidium bromide was nonrandom and favored sites such as that at 79 to 80.

The maltoporins with insertions at sites 79 to 80, 183 to 184, and 361 to 362 were produced in wild-type amounts (Fig. 4). In contrast, cultures containing pAM375 (insertion at 9 to 10) had lower amounts of protein in the outer membrane when grown above 37°C but wild-type amounts when grown at 30°C. This appears to be another *lamB* mutation which is at least partially temperature sensitive for synthesis or assembly in the outer membrane; the protein, once incorporated at 30°C, appeared to be stable at higher temperatures (results not shown). What steps in biogenesis are blocked in the two insertion mutants at higher temperatures is currently being studied.

The binding phenotype of the insertion at 183 to 184 is that of the wild type, and this mutation is essentially silent (Table 2). The maltoporins with insertions at 9 to 10 (produced at 30°C), 79 to 80, and 361 to 362 were all strongly starch binding negative, though only the insertion at 9 to 10 was affected in phage lambda sensitivity, even at the permissive temperature. These results are consistent with the roles of these regions predicted from models of maltoporin folding, considered below.

Influence of *lamB* mutations on the transport behavior of maltoporins. The influence of the substitutions and insertions on the pore properties of maltoporin were investigated by using lactose as a substrate, as previously described (13). Lactose is a nonmaltosaccharide which can be used to probe the nonselective pore in maltoporin. The mutations were transferred to the chromosomes of bacteria (to obtain stable, maltose-inducible *lamB* expression), and transport assays were carried out with strains lacking OmpF-OmpC pores (Fig. 4b). Compared with an isogenic, *lamB* wild-type strain, all of the maltoporin variants tested (Fig. 5) showed a decreased rate of lactose uptake. Even the 183-to-184 insertion, which did not affect starch binding, showed a 50% reduction in transport rates under conditions in which the levels of maltoporin expression in all of the strains were comparable (Fig. 4). The more strongly affected variants were, in addition to the Arg8→His variant described earlier (13), the insertion at 9 to 10 and the two strains with mutations near residue 360. The insertion at residue 119 to 120 exhibited a pore totally blocked towards lactose, suggesting a greatly decreased nonspecific pore size in this variant.

To test the maltodextrin selectivity of the channels formed, the influence of *lamB* alleles on dextrin fermentation was tested and the kinetics of maltose transport and its inhibition by maltohexaose were determined (Table 3). The growth results suggested that the pores in the 119-to-120 insertion mutant and, to a lesser extent, the 9-to-10 and 361-to-362 insertions were unable to permit permeation of longer maltodextrins. The other variants were able to channel dextrins at rates sufficient to permit dextrin fermentation.

The results of the kinetic analysis of transport in the strains with the variant maltoporins are shown in Table 3. Consistent with the notion that defects that affect the maltodextrin-binding site influence maltodextrin transport, there was a good correlation between the extent of the binding defect and the extent of the decrease in either maltose or maltohexaose transport affinity. However, some mutations caused differential decreases in maltose and maltohexaose affinity; for example, the Arg8→His substitution resulted in a greater loss of hexaose affinity, whereas the Arg360→Cys substitution resulted in a greater loss of affinity for maltose. Mutations that alter relative affinities for short and long dextrins have been noted earlier (7).

DISCUSSION

It is unusual, even in *E. coli* genetics, to have a plate assay which can sensitively discriminate between clones with subtle differences in the affinity of a binding site, yet this is exactly what the starch-swarm plate assay can do for maltoporin mutants. Clearly, this assay is, and will be, useful in the genetic definition of the maltodextrin-binding site in maltoporin. The phenomenon of inhibition of swarming by interaction with a macromolecule could be, in principle, used to study any surface receptor capable of such an interaction. The nature of the inhibition by starch was not

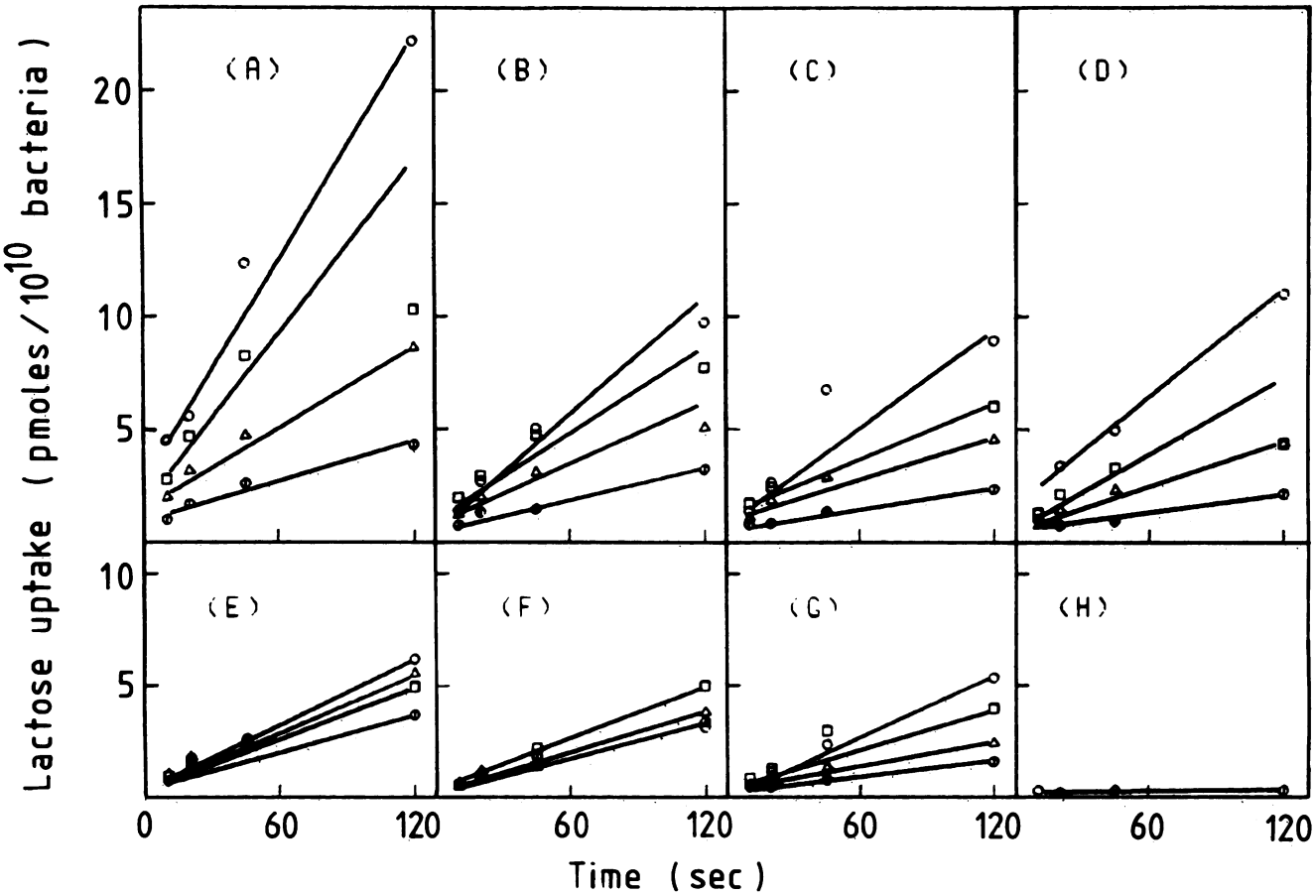

FIG. 5. Outer membrane permeability assayed by lactose transport. The following strains were used in these experiments (panels): A, BW2637 (*lamB*$^+$); B, BW2638 (*lamB1002*); C, BW2642 (*lamB153*); D, BW2645 (*lamB147*); E, BW2640 (*lamB1004*); F, BW2641 (*lamB146*); G, BW2646 (*lamB375*); H, BW2639 (*lamB203*). All strains contained an *ompR*::Tn*10* mutation to ensure that uptake of lactose was limited by maltoporin-mediated passage across the outer membrane. The concentration of bacteria used in each assay was 5×10^8/ml. Transport rates were measured in the absence of maltohexaose (○) and in the presence of 10^{-5} M (□), 10^{-4} M (△), and 10^{-3} M (◫) maltohexaose.

investigated, but microscopic observation of bacteria in the presence of starch indicated a complete lack of motility. It may be that starch or any macromolecule bound at the surface interferes with flagellar rotation.

Previous studies with a large number of mutants altered in maltoporin structure have led to a model of the folding of the protein across the outer membrane (Charbit et al., in press). This model was consistent with the orientation of residues that affect phage binding (on the surface of the protein), monoclonal antibody binding (surface), and starch binding (surface or transmembrane pore regions). Figure 6 shows a version of this model upon which the changes in the residues described in this paper have been superimposed. The discussion below considers the properties of the mutants in the context of this model.

The two-codon insertion at 9 to 10 caused both starch- and phage-binding defects, which is consistent with its predicted location on the outside of the membrane in the vicinity of residues 8 and 18. Substitution at the latter location produces a similar binding phenotype (Charbit et al., in press), confirming that the footprint of the lambda-binding site extends to this part of the protein. The reason that the insertion results in lack of protein in the outer membrane at higher growth temperatures is not known, though it is interesting that this insertion is in a region of the mature sequence predicted to have a spacer function in export (15). It is also feasible that this region of the protein is important for trimerization, since the mutation that changes residue 18 forms less stable trimers (B. Dargent, A. Charbit, M. Hofnung, and F. Pattus, J. Mol. Biol., in press).

The AspPro insertion at 79 to 80 specifically changes the starch-binding phenotype of maltoporin, as have substitutions at residues 74 and 82. This region in maltoporin, predicted to be in the transmembrane–deep-pore–periplasmic part of the protein, is clearly important for starch binding and transport. The *Bam*HI linker at this position in the gene will aid more detailed analysis of this region.

The insertion of a third tyrosine codon at position 119 to 120, where two Tyr codons already existed in wild-type protein, is in a region where substitutions were found to increase the affinity of starch binding (7, 18). The reduction in starch binding that resulted from the insertion was due to a loss of affinity, since lack of binding was also observed at the permissive temperature at which phage-binding was near normal. It is not clear why the mutation decreased the level of mature protein at the nonpermissive temperature; the defect may be in trimerization or, less likely, in synthesis and export.

The mutation that affects residue 194, causing phage resistance and starch-binding defects, is in a part of the gene

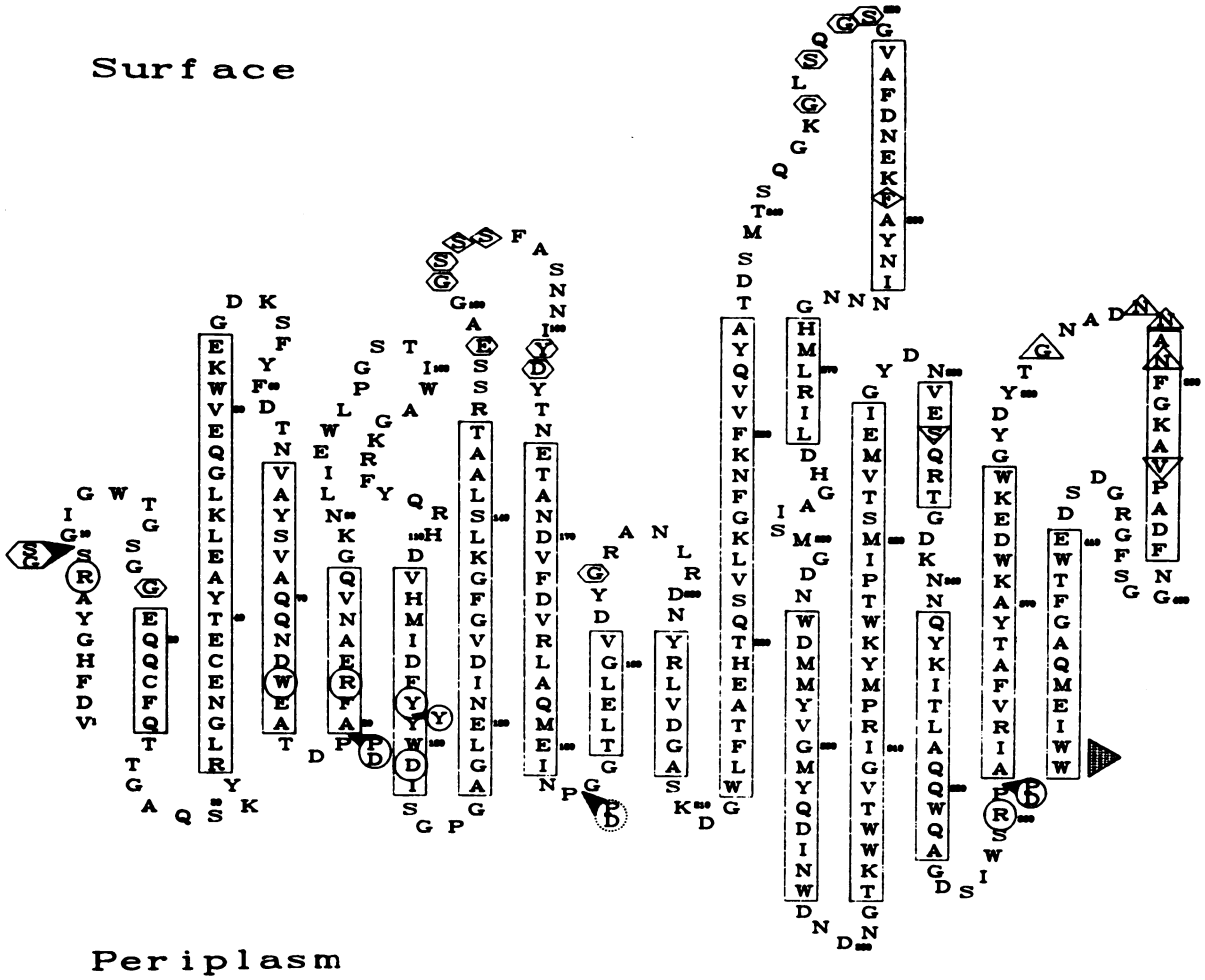

FIG. 6. Model of folding of maltoporin across the outer membrane of *E. coli*. The maltoporin sequence is shown with the one-letter amino acid code. The folding of this sequence with respect to the outer membrane is based on the information discussed by Charbit et al. (in press). Residues at which substitutions affect only the starch-binding site of maltoporin are circled; sites where substitutions affect both starch and lambda binding are surrounded by hexagons; sites that affect only lambda binding are surrounded by diamonds; sites that affect both lambda and monoclonal antibody binding (9) are surrounded by triangles (△); sites that affect only monoclonal antibody binding are surrounded by inverted triangles (▽). A dotted line surrounds the neutral insertion. The filled triangle is opposite the residues lacking in *lamB1007*. Filled arrowheads show the sites of insertions of the one or two residues indicated.

where no such substitution was previously found. It is consistent with the model (Fig. 6) that residue 194 is on the surface of the protein, where it could play a role in phage binding. Also, it should be noted that one of the few protease-accessible sites in the protein is near residue 200 (31), consistent with the external orientation of this region. The silent nature of the 183-to-184 insertion is also consistent with the model in that a change on the periplasmic side of the membrane, separated by a transmembrane region from 194, need not affect the binding phenotype.

The substitution at residue 360 and the insertion at 361 to 362 define a new region of the protein important of maltodextrin selective functions and shows that C-terminal residues are also significant in pore and binding site structure. The folding model shown in Fig. 6 would lead to the prediction that these residues are deep in the pore or at the periplasmic side of the outer membrane; this would be consistent with the result that these new mutations do not directly affect phage lambda binding. This is in contrast to substitutions and insertions of residues in the 380-to-390 region, which are clearly surface accessible (Charbit et al., in press). Overall, the results of this study are highly consistent wih the maltoporin topology predicted in Fig. 6.

The selectivity filter in the deep pore part of the maltoporin channel is affected by the nature of the residues at or near residues 8, 80, 120, and 360. It is striking that three of the single-amino-acid substitutions at these regions affect Arg residues (at 8, 82, and 360). These residues may interact with maltodextrins directly at the extended binding site (25). It will be of interest to test the accessibility of these residues in the pore directly by using biochemical approaches.

It is a striking reflection of the extreme perturbability of the biogenesis of maltoporin that a nonsense fragment that consists of 419 rather than 421 residues in the wild-type protein is found in much-reduced amounts in the outer membrane. The 419-residue protein, once synthesized, could clearly act as a phage lambda receptor and could support growth on dextrins, suggesting that the conformation of the residual protein in the outer membrane was not grossly affected. The one-codon insertion at 119 to 120 and the two-codon insertion at 9 to 10 both also caused defects at some point in maltoporin biogenesis at the nonpermissive

temperature. There is no additional evidence to suggest that these regions of the protein are specifically involved in macromolecular recognition or interactions, although these remain possibilities. It will be interesting to determine whether the reduction in protein levels in these mutants is due to poor synthesis, export, assembly of multimers, or increased protease sensitivity at some stage of biogenesis; these mutants should be useful for probing the little-understood later phases of maltoporin incorporation into the outer membrane.

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