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Fine structure mapping and complementation studies of the *metD* methionine transport system in *Salmonella typhimurium*

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Summary

A fine structure deletion map of the *metD* region of the chromosome of *Salmonella typhimurium* responsible for a high-affinity methionine transport system has been constructed. Complementation tests involving the introduction of *metD*$^+$DNA contained in a pUC8 vector into *metD* strains indicated the presence of four complementation groups in the *metD* region. This suggested that the methionine system belongs to the osmotic shock-sensitive class of transport system, and therefore should possess a periplasmic methionine-binding protein and several membrane proteins. But a deletion mutation covering all known *metD* point mutations did not affect the level of a methionine binding activity in osmotic shock fluids, suggesting either that the deletion did not extend into the gene encoding the binding protein, or that the binding activity is not associated with the *metD* system. Possible reasons for the failure to isolate mutations in the gene for the binding protein are discussed.

1. Introduction

L- and D-methionine are transported into *Salmonella typhimurium* by the *metD* high-affinity system (Ayling & Bridgeland, 1972; Ayling *et al.* 1979). This system was originally called the *metP* system, but was renamed *metD* (Shaw & Ayling, 1991), to conform with usage for the analogous system in *Escherichia coli* (Kadner, 1977; Bachmann, 1990). *metD* mutations result in reduced or negligible uptake of L- or D-methionine at low concentrations, and methionine auxotrophs carrying a *metD* mutation are, therefore, unable to grow on D-methionine: they can, however, still grow on the L-isomer because it enters through an independent low-affinity system (Betteridge & Ayling, 1975).

Bacterial transport systems for amino acids and similar molecules can be broadly divided into two major categories, shock-sensitive and shock-resistant. The shock-sensitive systems are complex and consist of from three to five genes, one of which encodes a binding protein located in the periplasm, and the others encode inner membrane proteins. In contrast, the shock-resistant systems are relatively simple and consist of a single gene encoding an inner membrane protein (Ames, 1986; Higgins *et al.* 1990). The *metD* system of *S. typhimurium* possesses some of the characteristics of the shock-sensitive class in that: (i) it is partially sensitive to mild osmotic shock; (ii) it is sensitive to inhibition by arsenate; and (iii) there is a binding activity for methionine in periplasmic shock fluids (Cottam & Ayling, 1989). Thus it would be expected that the *metD* region should contain from three to five genes organized into a single operon, rather than a single gene specifying an integral membrane protein. Recently, a fragment of *S. typhimurium* DNA which complemented *metD* mutations of *E. coli* was cloned in the lambda 1059 vector, and pieces of this fragment which were still able to complement *metD* mutations were sub-cloned in the pUC8 plasmid vector. Restriction maps of three plasmid sub-clones (pNS1, pNS4 and pNS5) have been constructed, and a protein of 34 kDa has been identified as the product of one of the genes (Shaw & Ayling, 1991). It is important to know how many genes are present in the *metD* region before attempting to sequence the DNA, and we have, therefore, constructed a fine structure genetic map of the *metD* region, and have used the pNS*metD*$^+$ plasmids to study the number of complementation groups in this region.

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Table 1. *Bacterial strains and their derivation*

Strain number	Genotype[a]	Source[b] or reference
	Salmonella typhimurium	
HU470	*metE205 glnP252 metD*$^+$	Ayling (1981)
HU593	*metE205 glnP252 metD1718 zaf-1351*::Tn*10 gal*	Cottam & Ayling (1989)
HU602	as HU593, but *metD1727*	Cottam & Ayling (1989)
HU603	as HU593, but *metD1728*	Cottam & Ayling (1989)
HU617–630	as HU470, but *metD2451–metD2464*	Spontaneous mutations
HU631–634	as HU470, but *metD2467–metD2470*	Spontaneous mutations
HU636–644	as HU470, but *metD2472–metD2480*	Spontaneous mutations
HU646–682	as HU470, but *metD2482–metD2518*	Spontaneous mutations
HU683	*metE205 glnP252 metD2453 leu*	From HU619, NG mutagenesis
TT38	*hisD8548*::Tn*10*	J. R. Roth
HU684	*metE205 glnP252 metD2453 leu hisD8548*::Tn*10*	TT38 × HU683, transduction
HU690–698	as HU470, but *metD2519–metD2527*	Spontaneous mutations
HU699	*metE205 glnP252 metD2453 leu hisD8548*::Tn*10 F′104 metD*$^+$*leu*$^+$	*E. coli* KL723 × HU684, conjugation
HU700–719	as HU699, *F′ metD2528–2547*	Spontaneous mutations
HU724	as HU470, but *metD2548*	Spontaneous mutations
HU725	as HU470, but *metD2549*	Spontaneous mutations
HU726	*metE205 metD760 gal recA*	Transductions[c]
SL5283	*metA22 metE551 trpB2 ilv-452 leu xyl-404 galE503 rpsL120 hsdL6 hsdSA29 hsdSB*	B. A. D. Stocker
TR2951	*hisD63 strA recA1*	C. F. Higgins
TT520	*srl*-202::Tn*10*	C. F. Higgins
	Escherichia coli	
KL723	*F′104leu*$^+$*metD*$^+$*/leuB6 recA13* (remaining genotype as in AB2463)	B. J. Bachmann and R. G. Lloyd (Low, 1972)

[a] Gene symbols are as in Sanderson & Roth (1988) for *S. typhimurium* and Bachmann (1990) for *E. coli*.
[b] In transduction or conjugation crosses, the donor is given first, and the recipient second.
[c] The *recA* mutation in strains HU726, etc. (listed in Table 2), was introduced by two-step transductions (Higgins *et al.* 1983), using phage grown on strains TT520 and TR2951 as donors, and the appropriate *metD* strains (listed in Ayling, 1981, or above), as recipients.

2. Materials and methods

(i) *Bacteria, phage and plasmids*

The bacterial strains used are listed in Table 1. The phage was P22 HT104/*int*. The following plasmids were derived by Shaw & Ayling (1991) from the ampicillin-resistant plasmid pUC8, and contained fragments of *S. typhimurium* chromosomal DNA (size of the inserts is shown in parentheses): pNS1 (1·9 kb), pNS4 (2·4 kb), and pNS5 (3·7 kb). The restriction maps for the inserts in these three plasmids contain sites for *Bam*H I, *Eco*R I, *Hinc* II, *Pst* I and *Sma* I (Shaw & Ayling, 1991).

(ii) *Growth conditions and media*

Bacteria were grown in nutrient broth for the mutant isolation and deletion mapping, and in either L-broth plus ampicillin at 50 μg/ml or liquid minimal medium plus ampicillin at 25 μg/ml for the plasmid studies. L-broth containing 0·2% galactose and 0·3% glucose was used when phage P22 was propagated on *gal* strains. Nutrient agar, L-agar and minimal agar were the respective liquid media solidified with 1·5% Oxoid No. 1 agar. Minimal agar plates were supplemented as follows (mg/ml): L-methionine, 20; D-methionine, 10; L-methionine-DL-sulphoximime (METX), 50; α-methyl-DL-methionine (AM), 500; vitamin B_{12}, 0·1; ampicillin, 25 (50 in L agar); tetracycline, 50.

(iii) *Isolation of* metD *mutants in a* metE *strain*

metE auxotrophs, besides being able to grow on L- or D-methionine, can also respond to vitamin B_{12}, which allows the synthesis of L-methionine via the alternative B_{12}-dependent *metH* pathway (Smith, 1971). Strain HU470 (*metE205 glnP252 metD*$^+$) was spread on minimal agar plates supplemented with vitamin B_{12} and either a mixture of METX and AM, or METX alone. *metD* mutations confer resistance to the analogue AM, which is transported only by the high-affinity system; strains carrying both a *metD* and a *glnP* mutation (the latter being a mutation in a high-affinity glutamine transport system) are also resistant to the second analogue, METX, which is thought to be transported by both the *metD* and *glnP* systems

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(Betteridge & Ayling, 1975). Resistant colonies were purified twice on nutrient agar, then tested for failure to grow on minimal agar + D-methionine. Only *metD* mutations prevent growth on D-methionine and confer resistance to these analogues (Betteridge & Ayling, 1975). *metD* deletion strains were identified as those strains which, when used as recipients in spot transduction against ten randomly selected *metD* point mutants as donors, produced two or more negative results; these negative results were confirmed using a whole plate for each transduction cross. Uptake of L-[^{14}C]methionine was determined using a rapid procedure (Ayling *et al.* 1979).

(iv) *Transduction and F′ transfer methods*

Spot transductions were performed by spreading cultures of *metD* recipient bacteria on minimal agar + D-methionine and then adding up to 9 drops of phage preparations propagated on the *metD* deletion mutants. Any cross which gave a negative result in the spot tests was repeated on a whole plate: these were done by mixing equal volumes of phage preparations and bacteria directly on the surface of the same plates. For the transduction of antibiotic resistance markers, equal volumes of phage and bacteria were mixed and incubated for 30 min at 37 °C, and aliquots spread on nutrient agar containing the relevant antibiotics. Mating methods to transfer the F′ 104 were based on those of Miller (1972).

(v) *Complementation analysis*

Plasmids were transformed into an r^-m^+ strain of *S. typhimurium* (SL5283) according to Lederberg & Cohen (1974), and then into *metD recA Salmonella* strains. Plasmids were also transduced into the latter strains with phage P22 (Sanderson & Roth, 1983). Transductants were selected on L-agar + ampicillin, purified by streaking out twice on the same medium, and overnight cultures were grown up from single colonies inoculated into L-broth + ampicillin. These cultures were diluted into fresh medium and grown for 1–2 h; they were then subcultured into minimal medium + L-methionine + ampicillin, and incubated overnight. The resulting cultures were then streaked onto minimal agar + D-methionine + ampicillin plates to detect complementation, and onto minimal agar + L-methionine + ampicillin as controls.

(vi) *Analysis of plasmid DNA*

Plasmid DNA was extracted using the methods of Birnboim & Doly (1979). Restrictions were carried out according to the manufacturer's instructions.

(vii) *Analysis of periplasmic proteins*

Cultures were grown in minimal medium + vitamin B_{12} or D-methionine. Shock fluids were prepared by the method of Nossal & Heppel (1966), modified by Higgins & Hardie (1983). Binding activity was determined by the dialysis method of Lever (1972). Wide-range (pH 3·5–9·5) and narrow-range (pH 4·0–6·5) isoelectric focussing gels were used according to the manufacturer's instructions (LKB Produkter). The pI of most amino acid-binding proteins lie within the latter range (Furlong, 1987).

3. Results and discussion

It had previously been shown that mutations in 14 strains which were resistant to the inhibitory methionine analogues α-methylmethionine (AM) or methionine sulphoximine (METX), and which failed to grow on D-methionine, mapped in the *metD* locus at min 6 on the *Salmonella* linkage map; all these mutations resulted in reduced transport of L- and D-methionine (Ayling *et al.* 1979; Sanderson & Roth, 1988). A deletion map has now been constructed and about 100 newly-isolated point mutations have been located on the map (Fig. 1). The *metD* region is divisible into a total of 19 deletion groups, each group of recipients giving a unique pattern of results when crossed with donor phage grown on the deletion strains. *metD2453* did not result in *metD*$^+$ recombinants with any of the *metD* point mutations tested, and so was presumed to be a deletion extending through the whole of the *metD* region so far mapped. *metD2459* appears to be a double deletion. *metD1708* is a deletion covering the mutations *metD760*, *1707*, *766*, *761*, and *762*, but not *1711*, *767* or *1710*, confirming the results of Ayling *et al.* (1979).

To perform a complementation analysis, plasmids pNS1, pNS4 and pNS5 were introduced into each of 13 strains containing *metD* mutations distributed across the genetic map (Fig. 1), and the results are shown in Table 2. The strains also contained a *recA* mutation, to prevent recombination between the plasmid insert and the homologous region on the chromosome. Only plasmid pNS1 could be introduced by transformation, whereas all three plasmids could be introduced by transduction. In the case of transduction, it was found that many of the transductants would not grow when streaked directly from L-agar to minimal agar. Growth of the transductants in broth and minimal medium, however, produced cultures that grew well on minimal agar; this method was, therefore, used to produce cultures for complementation tests.

Plasmid pNS1 complements only three mutations, which thus defines one complementation group. Plasmid pNS5 complements an additional mutation, suggesting the presence of a second group, and plasmid pNS4 complements a further four mutations, indicating a third group. Although *metD1711* maps in deletion group 12, between *metD2513* (deletion group 11) and *metD767* (deletion group 14), it is not complemented by pNS4, and so is probably a polarity mutation. Plasmid pNS4, which apparently contains

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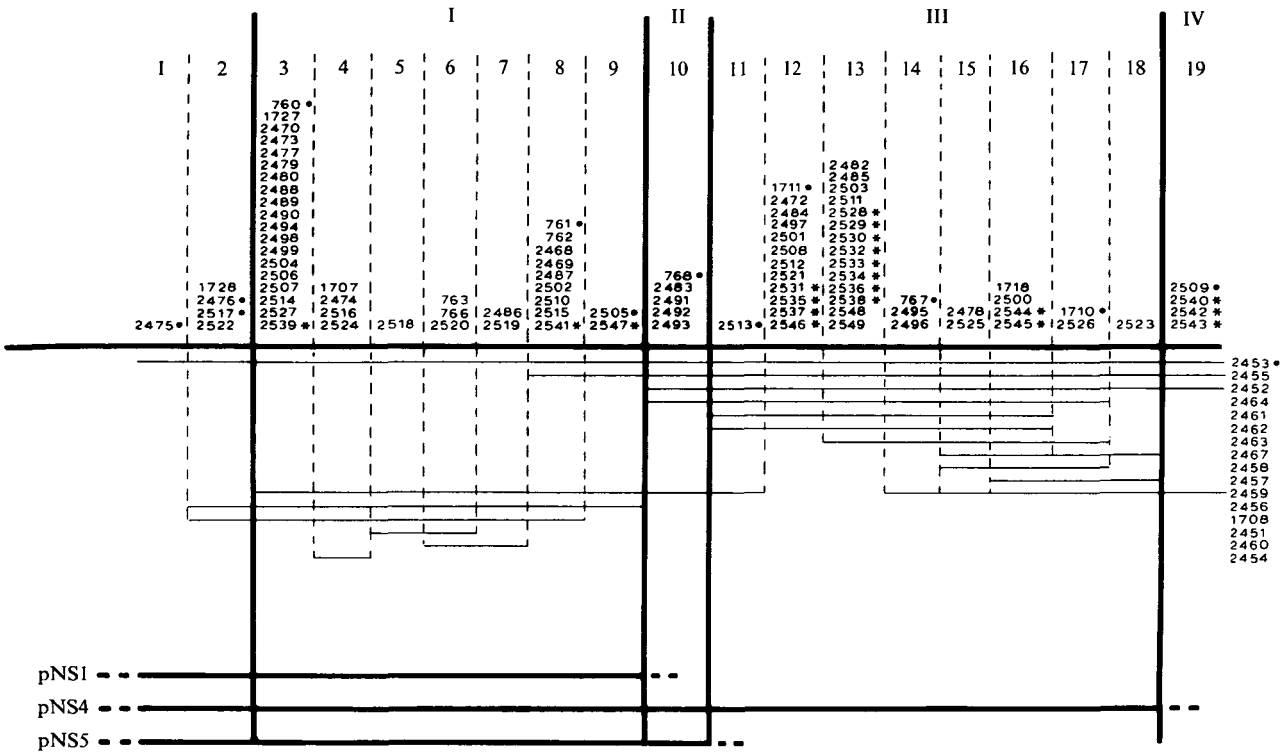

Fig. 1. Deletion map of the *metD* region in the *Salmonella* chromosome. The thick horizontal line in the middle of the Figure represents the *metD* region of the chromosome. The thin horizontal lines below the chromosome represent the extent of 16 *metD* deletions, the numbers of which are shown on the right-hand side. The numbers in columns in the upper half of the Figure are the *metD* point mutations used as recipients in crosses against donor deletion strains. The *metD* mutations on the F′ in strains HU700–719 are indicated thus: ★. The *metD* mutations tested for complementation in Table 2 are indicated thus: ●. The numbers 1–19 at the tops of the columns are the numbers of the deletion segments defined by the ends of the deletions. Columns 1 and 2 may be the site of the promoter region (Shaw & Ayling, 1991). The relative size of each segment is unknown. The heavy horizontal lines at the bottom show the probable extent of *metD* DNA inserts contained in plasmids pNS1, 4 and 5, as deduced from complementation tests; the exact end points are unknown (the sizes of the inserts are not to scale). The heavy vertical lines represent the boundaries of the groups determined by the complementation analysis in Table 2; the complementation groups are identified by Roman numerals at the top of the Figure.

the most *metD* DNA (but see below), does not complement the largest deletion (*metD2453*), therefore there must be at least one additional complementation group not present on the cloned *metD* DNA.

Three mutations in deletion groups 1 or 2 were not complemented by any of the plasmids; these might be mutations in the promoter region or chain termination mutations with strong polar effects, such that none of the genes in the operon were expressed. Shaw & Ayling (1991) have suggested that the *metD* promoter region is present on all the plasmids, at the left hand side of the map in Fig. 1. The transport of L-methionine in six mutants (*metD2475*, *760*, *768*, *2513*, *2523* and *2509*), was shown to be less than 5% of the *metD*$^+$ parent strain HU470 (1·44 nmol min^{-1} mg^{-1} dry weight). As shown in Fig. 1, these mutations occur in each of the complementation groups and control region, indicating that all the groups identified here are involved in the transport process.

To investigate why transductants grew on solid supplemented minimal agar only after growth in L-broth and minimal medium, plasmid DNA was extracted from an isolate of strain HU730 (*metD768*) containing pNS5 which was unable to grow on minimal agar + L-methionine, and from another isolate of the same strain which, after growth in L-broth and minimal agar + L-methionine, was able to grow on the latter medium (as shown in Table 2). It was found that there is less than 5% of the amount of plasmid DNA present in the cells which are able to grow on minimal medium + L-methionine compared with cells unable to grow on this medium. It seems probable, therefore, that the plasmid insert has a deleterious effect on cells when present in high copy number, and that there has been a selection during growth in broth and minimal medium for reduced plasmid copy number.

According to Shaw & Ayling (1991), plasmid pNS5 contained a larger amount of insert DNA than the two other plasmids, but the complementation results in Table 2 do not support this conclusion. There was no significant difference in the amount of DNA present from restrictions of plasmid pNS5 compared with those of Shaw & Ayling (1991) (results not shown). It is possible that two unrelated pieces of DNA from the initial lambda *metD*$^+$ phage could have been joined together in the pUC8 vector, i.e. although pNS5 contains 3·7 kb of *S. typhimurium* DNA, only

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Table 2. *Complementation tests with plasmid-containing strains*

Recipient[b]	Plasmid[a] pNS1	pNS4	pNS5	Complementation group
HU735 (*metD2475*)	–	–	–	Promoter?
HU736 (*metD2476*)	–	–	–	
HU740 (*metD2517*)	–	–	–	
HU726 (*metD760*)	+[c]	+	+	I
HU727 (*metD761*)	+[c]	+	+	
HU737 (*metD2505*)	+	+	+	
HU730 (*metD768*)	–[c]	+	+	II
HU741 (*metD2513*)	–	+	–	III
HU732 (*metD1711*)	–[c]	–	–	
HU729 (*metD767*)	–[c]	+	–	
HU731 (*metD1710*)	–[c]	+	–	
HU739 (*metD2509*)	–	+	–	
HU733 (*metD2453*)	NT[d]	–	NT	

[a] Plasmids pNS1, pNS4 and pNS5 were introduced by transduction on L-agar. After growth in L-broth and minimal medium + L-methionine, isolates were tested on minimal agar + D-methionine (or L-methionine as a control). All media contained ampicillin, to ensure the presence of the plasmids.
[b] Location of the *metD* mutations is shown in Fig. 1.
[c] The same result was observed when the plasmid was introduced by transformation.
[d] NT, not tested.
+, Complementation; –, no complementation.

about half of this is *metD* DNA. Alternatively, pNS5 might contain a mutation which inactivates the distal *metD* gene(s).

A further complementation analysis was attempted using an F′ plasmid complementation system, based on the rationale of Hogarth & Higgins (1983). F′ 104 (*metD*$^+$*leu*$^+$) was transferred from *E. coli* strain KL723 to strain SL5283, a *Salmonella* 'intermediate' strain, and thence into strain HU684 (*metE205 glnP252 metD2453 leu hisD8548*::Tn*10*), to produce strain HU699. This strain was able to grow on D-methionine and was sensitive to the analogue METX, showing that the *metD2453* mutation is recessive. A new set of METX-resistant mutants unable to grow on D-methionine was then selected from strain HU699. Since the chromosomal deletion mutation *metD2453* extends throughout the whole *metD* region in which mutations occur (Fig. 1), resistance to METX must have occurred by a *metD* mutation on the F′. Twenty such F′ mutants, HU700–HU719 (containing mutations *metD2528–metD2547*, respectively, see Fig. 1) were then used as donors against recipients HU638 and HU659, carrying chromosomal mutations *metD2474* and *metD2495* in deletion groups 4 and 14, respectively. None of the F′s carrying a *metD* mutation were able to complement the chromosomal *metD* mutations, indicating a single complementation group. The F′ *metD* mutations were shown by transduction analysis to be distributed across the *metD* region (Fig. 1), and it was confirmed that the F′ was present and was being transferred to recipient strains.

The above analysis suggests that complementation cannot occur in the *metD* system between a wild-type gene on the chromosome and one on the F′ plasmid. Ames & Nikaido (1978), using a similar F′ system, also failed to detect complementation between mutants with defective membrane components in the periplasmic histidine transport system of *S. typhimurium*, which is encoded by four genes (Higgins *et al.* 1982), whereas Hogarth & Higgins (1983) obtained complementation between oligopeptide transport mutants of *S. typhimurium*. The reason for the difference between these systems is not known.

Cottam & Ayling (1989) were unable to detect any differences between the periplasmic proteins from a *metD*$^+$ strain and several *metD* mutant strains on one-dimensional polyacrylamide SDS gels. Iso-electrofocussing gels, on which more discrete bands are obtained were, therefore, tried. However, no difference was observed between the periplasmic proteins in the osmotic shock fluids from strain HU470 (*metD*$^+$) and HU619 (*metD2453*, deleted for all the known *metD* region, as shown in Fig. 1), even when the strains were grown in minimal medium + D-methionine, conditions which should result in derepression of the synthesis of the transport system. Similarly, no significant difference was found in the binding activity for L-methionine in strain HU470 (59 pmol/mg protein) and strain HU619 (68 pmol/mg protein).

Three possibilities could explain the failure to find mutants lacking a methionine binding protein or a methionine binding activity: (i) the *metD* system does not contain a binding protein (i.e the methionine binding activity detected does not belong to the *metD* system; (ii) a binding protein does exist, but is not detectable as a discrete band on one-dimensional gels; or (iii) the section of the *metD* region in which mutations have been detected does not include the binding protein gene. This third explanation seems quite likely, because it is possible that the method used for selection of the *metD* mutants, i.e. that of analogue resistance coupled with an inability to grow on D-methionine, does not select for mutations in the binding protein gene. This would be the case if there were two binding proteins, each capable of passing the methionine analogue on to the *metD* membrane proteins; there is indirect evidence to support this idea (Cottam & Ayling, 1989). The situation in the *metD* transport system may parallel that which has recently been found in the sulphate/thiosulphate transport system in *E. coli* and *S. typhimurium*. The 'cysA' region involved in sulphate transport was found to contain three complementation groups from abortive transduction analysis (Mizubuchi *et al.* 1962), and a sulphate binding protein was later isolated (Pardee *et al.* 1966); these complementation groups have now been shown to correspond with the three genes for

membrane transport proteins (Sirko *et al.* 1990); a novel thiosulphate binding protein gene has been identified immediately upstream from the 'cysA' region (Hrynkiewicz *et al.* 1990), but mutations in the sulphate binding protein gene could not be isolated (Ohta *et al.* 1971), and the gene for the sulphate binding protein remains to be identified (Sirko *et al.* 1990). In the case of the methionine transport system, similar direct evidence, such as that from the DNA sequence of the *metD* system, is required to confirm the theory that the *metD* system is indeed periplasmic in nature, and to identify the genes for the methionine binding protein(s) and the membrane proteins.

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