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VARIANT FORMS OF MATRIX PROTEIN IN *ESCHERICHIA COLI* B/r BEARING N PLASMIDS

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Summary

Plasmids of the N incompatibility group have been found to decrease or virtually eliminate the synthesis of the 36 500 dalton outer membrane matrix protein of their *Escherichia coli* B/r hosts (Iyer, R. (1977) Biochim. Biophys. Acta 470, 258–272 and Iyer, R., Darby, V. and Holland, I.B. (1978) FEBS Lett. 85, 127–132) or modify its composition. Although the 34 000 dalton tol G protein is slightly increased in some strains, it is identical in composition to the homologous protein from the plasmidless host. In three of five N^+ strains, the synthesis of the modified matrix proteins depends on the temperature of cultivation of the strains in which they occur. The alterations to the matrix proteins are non-identical and do not affect the expression of several plasmid-coded functions including those of sensitivity to the N plasmid-specific filamentous bacteriophage IKe (Khatoon, H. and Iyer, R. (1971) Can. J. Microbiol. 17, 669–675), or their interbacterial transfer via conjugation to appropriate recipient strains. Thus, although the significance of the variant matrix proteins in N^+ strains with respect to plasmid-mediated functions remains unclear, N plasmids nevertheless provide a convenient system which might be used to elucidate the events that precede the insertion of this protein into the outer membrane of *E. coli* B/r hosts.

Introduction

The introduction of each of seven plasmids obtained from independent sources and belonging to the incompatibility group N in the host strain *Escherichia coli* B/r and the alterations they mediate in the integral membrane

Abbreviation: SDS, sodium dodecyl sulfate.

proteins of the respective transconjugants thus obtained, have been reported: whereas in the transconjugants bearing the plasmids rR45, rR46, rR199 and rR205, the level of the matrix protein or 'porin' (interchangeably used terms) is not affected, it is virtually eliminated in strains bearing rR48, rRM98 and rR269 [1]. In the case of rRM98, we have also found a variable decrease in the level of porin when membranes from independently isolated transconjugants derived from the same parental cross are compared [2].

The matrix protein is a peptidoglycan-associated integral outer membrane component of mol. wt. 36 500 [4–6]. It constitutes passive hydrophilic pores which facilitate the rapid diffusion of low molecular weight substrate molecules [7]. It is present in about $1.1 \cdot 10^5$ copies/cell and its properties and those of other membrane proteins have been comprehensively reviewed recently [8].

We describe here the peptide patterns obtained following the limited proteolysis of porin obtained from isogenic N^+ and N^- strains. With the exception of the $rR205^+$ strain, porin-synthesizing strains bearing each of four different N plasmids have been found to contain variant forms of this protein which produce non-identical sets of polypeptides. In three of these strains, porin synthesis is temperature dependent and occurs only if the cells are grown at 37°C.

Materials and Methods

Bacterial strain. The derivation of the strains, the relevant markers they bear and the sources of the plasmids have been previously described [1,2]. The level of porin associated with each of them is summarized in Table I.

Enzymes. Trypsin (bovine, type XI, DCC treated, crystalline), α-chymotrypsin and protease (*Staphylococcus aureus* V8) were purchased from Sigma Biochemical Company, Worthington Biochemical Corporation and Miles Laboratories Ltd., respectively.

Media. The strains were maintained as described earlier [9] and cultivated

TABLE I

BACTERIAL STRAINS AND PORIN LEVELS IN THEIR OUTER MEMBRANES

Strain	Level of porin
LEB18	wild type
LEB500(rRM100)	absent
LEB501(rRM98) *	absent
LEB502(rRM98)	absent
LEB503(rRM98)	visibly reduced
LEB18(rRM98)	wild type
LEB508(rR45)	wild type
LEB509(rR46)	wild type
LEB505(rR48)	absent
LEB510(rR199)	visibly reduced
LEB506(rR269)	absent
LEB511(rR205)	wild type
JE2571	wild type
JE2571(rRM98)	wild type

* Although the 36 500 mol.wt. protein was absent, a heavier protein of apparent mol.wt. 37 500 appeared as a major band in this strain.

prior to membrane isolation on either L broth or Davis and Mingioli minimal broth as indicated.

Preparation of outer membranes. Cells grown to an absorbance of 0.4–0.5 unit at 650 nm were chilled and harvested by centrifugation. Total envelopes from cells resuspended in 10 mM sodium phosphate buffer (pH 7.2) were obtained by sonication using a model W-350 Sonifier (Branson Ultrasonics Corp.) and a 5/8 inch chilled probe. Sonication was performed in 4–6 30-s pulses separated by 1 min rest intervals. The cells were chilled in an ice/water mixture throughout. Unbroken cells were removed by low speed centrifugation. The recovery of total and outer membranes following the solubilization of cytoplasmic membranes was as described by Filip et al. [10].

Gel electrophoresis and limited proteolysis procedures. The preparation of membrane samples for electrophoresis was as described before [1]. Outer membrane preparations corresponding to 50 μg protein/lane were predigested in sample buffer at 100°C for 5 min and electrophoresed on 9% acetylamide gels. After staining with Coomassie brilliant blue, protein bands of interest were cut and digested for 30 min, without prior elution, in the stacking gel of a second gel which contained 15% acrylamide in the separating gel. Digestion was carried out at room temperature; consequently, peptide patterns of test porins should be compared only with controls appearing on the same gel. Undigested controls were always included to confirm the purity of the proteins. The procedures for staining and proteolysis were essentially as described by Cleveland et al. [11].

Results

Comparison of outer membrane proteins from E. Coli B/r and its rR45$^+$, rR46$^+$, rR199$^+$ and rR205$^+$ transconjugants

The products of limited proteolysis of porin from the strains being compared are presented in Fig. 1. All strains were grown in L broth at 37°C. The polypeptides obtained from digestion of porin from LEB508(rR45) with chymotrypsin and protease (lane i in Fig. 1A and B, respectively) are nearly identical to those of the corresponding controls (lane iii); any differences in this case are seen in the tryptic patterns (Fig. 1C) in which the rR45$^+$ porin lacks several polypeptides seen in the control lane. None of the enzymes used show any differences between the patterns obtained with LEB511(rR205) and the control (compare lanes v and iii in Fig. 1). Porins from LEB509(rR46) and LEB510 (rR199) (lanes ii and iv, Fig. 1) are significantly altered relative to the control porin. In addition to a few new polypeptides, some bands are either absent of significantly decreased. The variant forms of porin seen in the rR45$^+$, rR46$^+$ and rR199$^+$ strains are also non-identical to each other. With the exception of LEB509(rR46), differences in porin composition are relatively less obvious if cultures are grown in minimal broth. A similar analysis of the tol G proteins from each of these five strains shows no differences in polypeptide composition when cultures are grown in either L broth or minimal broth.

Comparison of outer membrane proteins from E. coli and two transconjugants bearing rRM98

Electrophoretic analysis by SDS-polyacrylamide gel electrophoresis of the

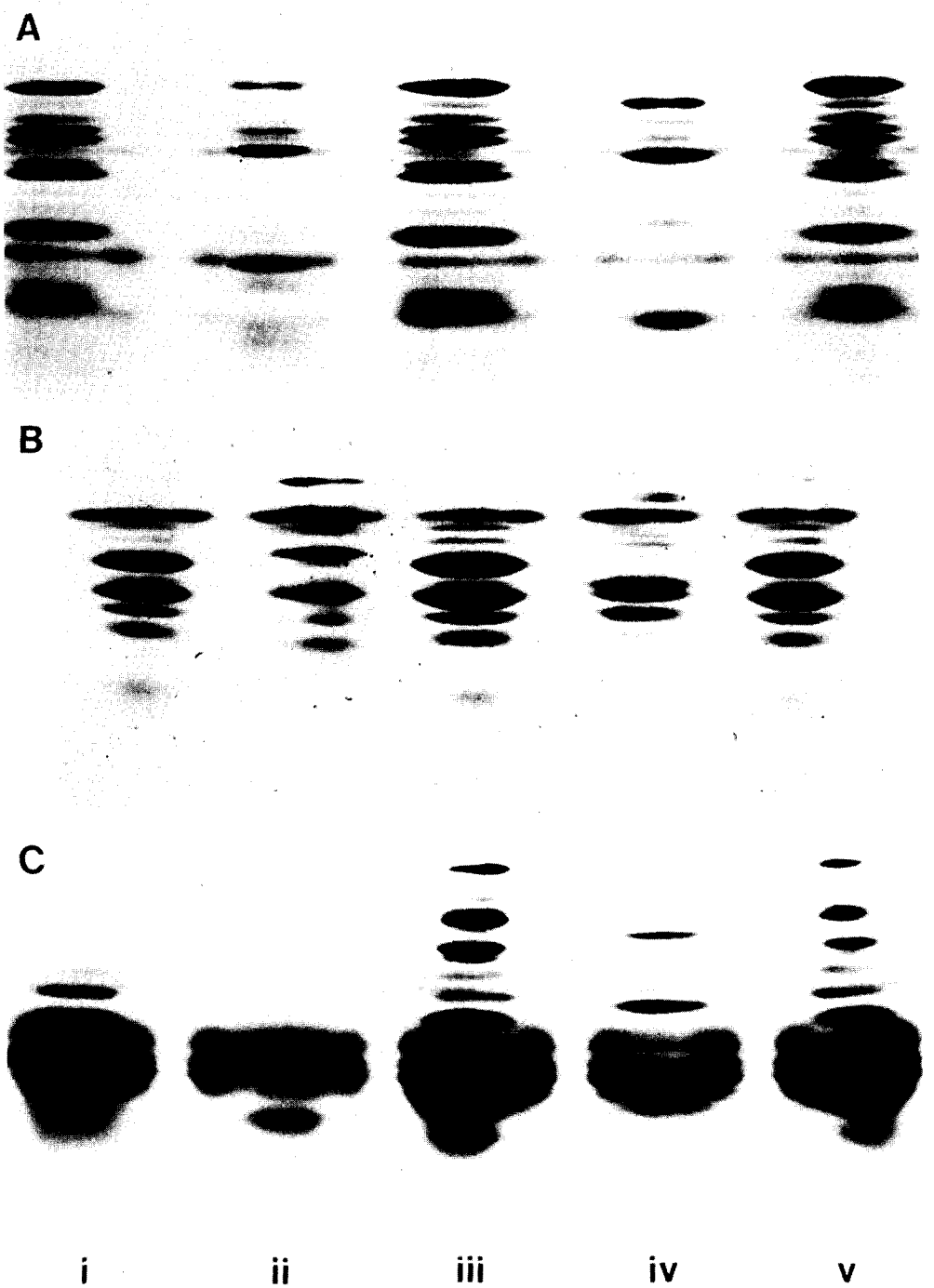

Fig. 1. Limited proteolysis of porin from outer membranes of (i) LEB508(rR45); (ii) LEB509(rR46); (iii) LEB18; (iv) LEB510(rR199); (v) LEB511(rR205). (A) Chymotrypsin, 10 μg/lane. (B) Protease, 1.2 μg/lane. (C) Trypsin, 20 μg/lane. Outer membranes were isolated fron sonicated cells grown to exponential phase in L broth at 37°C, as sarkosyl-insoluble material [10]; porin was separated on SDS-polyacrylamide gels containing 9% (w/v) polyacrylamide in the separating gel. Enzyme digestion was done on porin in gel slices as described in [11].

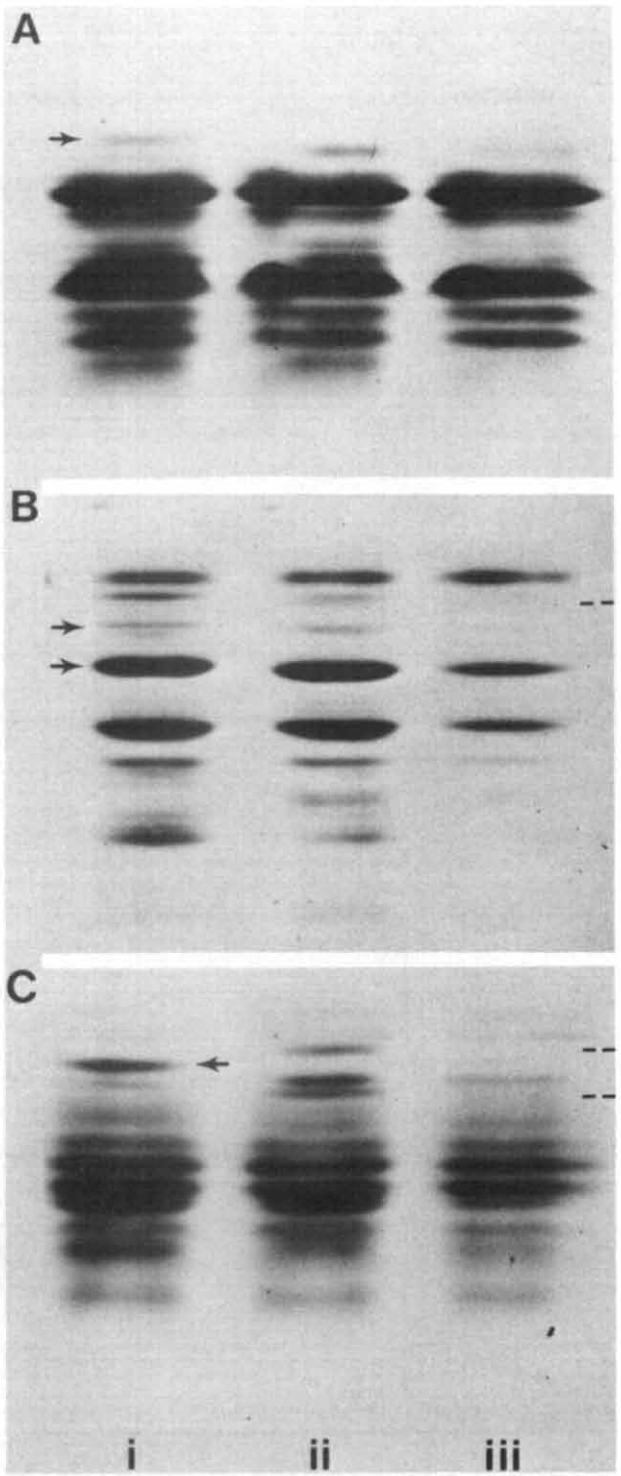

Fig. 2. Limited proteolysis of porin from outer membranes of (i) LEB501(rRM98); (ii) LEB18; (iii) LEB503(rRM98). Details as in Fig. 1. Arrows indicate heavier peptides in lane (i) relative to those in lane (ii). - - - - -, absence of peptides in lanes (i) and (iii) relative to those in (ii).

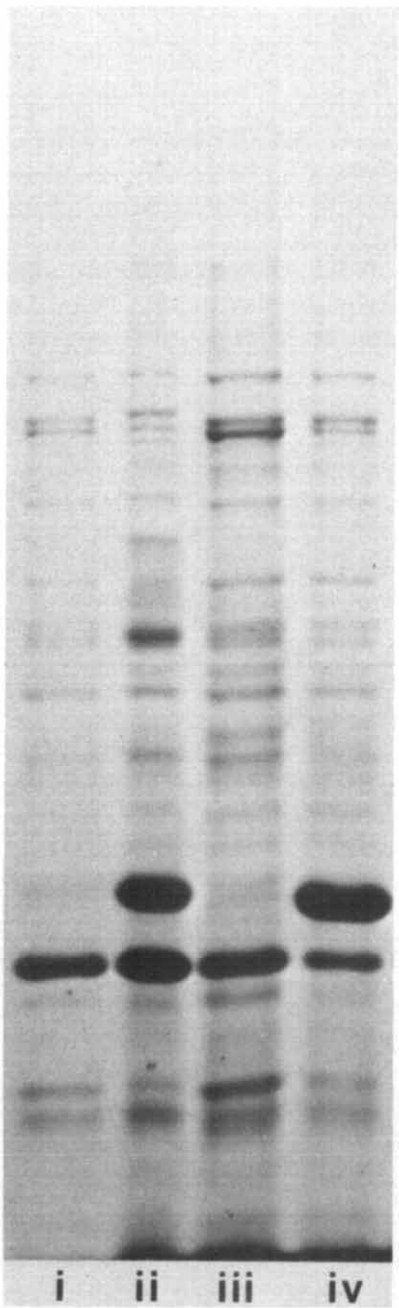

Fig. 3. SDS-polyacrylamide gel electrophoresis of total envelopes from (i) LEB508(rR45); (ii) LEB509(rR46); (iii) LEB510(rR199); (iv) LEB511(rR205). Cultures were grown to exponential phase in L broth at 23°C and total envelopes obtained following sonication. The concentration of polyacrylamide in the separating gel was 9% (w/v).

outer membranes from several rRM98$^+$ transconjugants of *E. coli* B/r which were derived from the same conjugative cross has indicated that the level of porin shows considerable variation ([1]; Whitwill, S.T. and Iyer, R., unpublished data). In the case of the strain LEB501(rRM98), a slower migrating protein of apparent mol. wt. 37 500 replaces the 36 500 dalton protein. Whereas in LEB503(rRM98), the 36 500 dalton porin is visibly reduced, it is synthesized in wild-type amounts in LEB18(rRM98) [2]. The latter strain grows erratically at 37°C, although it was originally isolated at this temperature. Consequently, we have compared the 37 500 and 36 500-dalton proteins from LEB501(rRM98) and LEB503(rRM98), respectively, with porin from the control strain LEB18. These preparations were obtained from cultures grown in L broth at 37°C.

The products of proteolysis of porin from strains LEB501(rRM98) and LEB503(rRM98) are compared with that from LEB18 in Fig. 2, lanes i, iii and ii, respectively. A comparison of lanes i and ii clearly shows the presence of one slow moving peptide in Fig. 2A and C whereas two such components one of which is a major one, can be seen in Fig. 2B (indicated by arrows); a few minor peptides are either missing and some new ones can also be seen. Relatively few but clear differences between LEB503(rRM98) and LEB18 (lanes iii and ii) are observed: chymotrypsin digestion (Fig. 2A) shows the absence of the fastest migrating peptide in lane iii; whereas many minor peptides are reduced or missing when protease digests are compared (Fig. 2B), at least two peptides which are present in moderate concentrations in lane ii are altogether absent in lane iii. Porins from both rRM98$^+$ strains are similar in that they lack a few peptides which appear in the control lane; these are indicated by broken lines Fig. 2B and C). These results have also been confirmed using porins from cultures grown at 23°C in L broth, although the differences are clearer at the higher growth temperature.

A comparison of the tol G proteins from strains grown at both temperatures shows no differences from the control protein. A similar analysis of the matrix and tol G proteins from the *E. coli* K12 strain JE2571 and its rRM98$^+$ transconjugant shows no differences between them. A few minor differences in some minor envelope proteins in these two strains have been described elsewhere [12].

Temperature-dependent synthesis of porin in some N$^+$ transconjugants

LEB18(rRM98) is more easily cultivated at 23°C rather than at 37°C (see above). However, at the lower temperature, porin is not synthesized/integrated into the outer membrane of this strain. The membranes of four other porin-synthesizing N$^+$ strains have been similarly examined after cultivation of the cells at 23°C in L broth (Fig. 3). In the transconjugants bearing the plasmids rR45 (lane i) and rR199 (lane iii), this protein is significantly reduced if not virtually eliminated whereas in the rR46 (lane ii) and the rR205$^+$ (lane iv) strains, its level does not appear to be altered. The level of porin in LEB18 cultivated at both temperatures is comparable (data not shown).

Discussion

The outer membrane of *E. coli* and possibly other Gram-negative bacteria serves as a permeability barrier and provides receptors for colicins, bacteriophages and vitamins. In the case of strains bearing transmissible plasmids, they must, in addition, provide specific sites through which gene transfer can occur. This event is most likely preceded by the specific attachment of recipient cells to the surface of donors. The principal outer membrane components are proteins, phospholipids and lipopolysaccharides. A few distinct proteins constitute a major fraction of the total outer membrane proteins and include porin, the tol G protein and lipoprotein. Mutants lacking one or more of these proteins have been isolated by various selective procedures and these have proved to be considerably useful in elucidating their physiological role [8] and in assessing the significance of the tol G protein in conjugating *E. coli* cells [13]. Mutants lacking porin but which contain instead a higher mol. wt. protein have been described [14—16] as also have those which have an altered tol G protein [17].

Our studies with the N incompatability group of plasmids have shown that in *E. coli* B/r transconjugants bearing some N plasmids, the matrix protein is either virtually eliminated [1] or variably reduced [2], or as described above, has an altered polypeptide composition. Limited proteolysis of porin obtained from the F'lac$^+$ derivative of LEB18 and one spotaneous 'plasmid-cured' derivative of LEB505(rR48) in which the level of this protein is fully restored [2] shows complete identity with the control protein (data not shown). These changes in the level or composition of porin do not seem to interfere with the phenotypic expression of plasmid-coded resistance to one or more antibiotics, sensitivity to the filamentous bacteriophage IKe or (plasmid) autotransmissibility to an appropriate recipient strain. Very interestingly, too, the modified matrix proteins in the rR45$^+$, one rRM98$^+$ and the rR199$^+$ strains are synthesized only if these strains are cultivated at 37°C but not if they are grown at 23°C. These observations suggest that N plasmids might provide a convenient system in elucidating the molecular events which determine the posttranslational processing of porin.

The insertion of proteins into the outer membranes of bacteria is preceded by their synthesis, translocation across the cytoplasmic membrane and murein layer, and the posttranslational modification of the growing polypeptide chain. Strong evidence supporting the possibility that these events occur simultaneously has been presented [18]. Studies on the processing of prolipoprotein, a precursor of lipoprotein, have also indicated that the translocation and modification of this protein across the cytoplasmic membrane are tightly coupled [8]. This model is also probably applicable to the matrix and the tol G proteins, precursors for both of which have been identified [16].

The translocation and assembly of membrane proteins has been studied in unsaturated fatty acid auxotrophs. These studies have demonstrated that membrane fluidity also plays an important role. Ito et al. [19] have reported a more stringent requirement for a fluid membrane for the insertion of outer rather than inner membrane proteins. DiRienzo et al. [8] have found differences in the assembly of the outer membrane proteins under conditions of a crystalline membrane state: whereas lipoprotein assembly is not affected, the assembly of

the matrix protein is completely inhibited. The possibility that the elimination or decreased levels of the matrix protein in rRM98$^+$ strains [2], and in the rR45$^+$, rRM98$^+$ and rR199$^+$ strains following their cultivation at 23°C (see above), might be due to plasmid-determined alterations in membrane fluidity is being examined.

The absence of the matrix protein in some N$^+$ transconjugants could be due to (a) failure to synthesize its precursor, the promatrix protein; or (b) an improper proteolytic processing of the latter substrate due to alterations in the activity of or target sites used by intracellular protease(s); or (c) a synthesis of intracellular inhibitors which might variably inactivate the protease(s) or act at the level of translation or transcription, or (d) altered membrane fluidity.

The fact that plasmids like rRM98 cause the elimination or a variable reduction in the level of the matrix protein [2] rules out the possibility (a) and supports (b) and/or (c). The identification of variant forms of matrix protein in the case of four N$^+$ strains can be explained on the basis of (b). It is interesting that in the case of rRM98 at least, the various effects on the matrix protein are seen in the B/r but not in the K12 host. Analysis of membranes from transformants of JE2571 bearing plasmid DNA from LEB500(rRM100), LEB501(rRM98) and LEB505(rR48) has confirmed the presence of wild-type levels of porin in this host. Differences in response to protease inhibitors which affect porin production in the K12 and B strains of *E. coli* have been reported recently [20]. Thus it is possible that should variations in porin levels described above be due to plasmid-coded intracellular inhibitors as suggested in (c) above, these effects fail to be expressed in the K12 background.

It may also be significant that N plasmids specifically modify the matrix but not the tol G protein in the B/r host. On the basis of this observation, the existence of two independent sets of processing enzymes which specifically act on the precursors of the matrix or tol G proteins as substrates might be postulated.

Note added in proof (Received June 22nd, 1979)

The strains LEB18(rRM98), LEB508(rR45) and LEB510(rR199) which have synthesized modified porins at 37°C since their isolation in 1975 have recently been found to fail to do so. This has also been observed independently by Dr. I.B. Holland, University of Leicester, England. Although the reason for this spontaneous change is not understood, we have found significant alterations in the fatty acid levels of these and other N$^+$ strains (Iyer, R., et al., manuscript submitted to Nature). It is possible that these alterations might be implicated somehow in the nonintegration of modified porin and possibly in other as yet urecognized effects.

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