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Stationary Phase Expression of a Novel *Escherichia coli* Outer Membrane Lipoprotein and Its Relationship with Mammalian Apolipoprotein D

IMPLICATIONS FOR THE ORIGIN OF LIPOCALINS*

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We report a novel outer membrane lipoprotein of *Escherichia coli*. DNA sequencing between *ampC* and *sugE* at the 94.5 min region of the *E. coli* chromosome revealed an open reading frame specifying 177 amino acid residues. Primer extension analysis demonstrated that the promoter is activated at the transition between exponential and stationary growth phases under control of the *rpoS* sigma factor gene, and this was confirmed *in vivo* by monitoring expression of β-galactosidase activity from a *lacZ* translational fusion. The amino acid sequence exhibited 31% identity with human apolipoprotein D (apoD), which is a component of plasma high density lipoprotein and belongs to the eukaryotic family of lipocalins. The bacterial lipocalin (Blc) contained a short deletion of 7 amino acid residues corresponding to a hydrophobic surface loop that is thought to facilitate the physical interaction between apoD and high density lipoprotein. However, Blc exhibited a typical prokaryotic lipoprotein signal peptide at its amino terminus. Overexpression, membrane fractionation, and metabolic labeling with [^{3}H]palmitate demonstrated that Blc is indeed a globomycin-sensitive outer membrane lipoprotein. Blc represents the first bacterial member of the family of lipocalins and may serve a starvation response function in *E. coli*.

The lipocalin superfamily consists of widely distributed, primarily extracellular, eukaryotic proteins that bind and transport small hydrophobic ligands (1). The molecular structures of four lipocalins (plasma retinol-binding protein (2), bilin-binding protein (3), insecticyanin (4), and α-lactalbumin (5)), revealed a common structural motif that consists of an eight-stranded antiparallel β-barrel, arranged as two stacked orthogonal sheets, with a COOH-terminal α-helix. Despite the common lipocalin fold, only 25–30% amino acid sequence identity exists between lipocalins of known structure (6). The cup-shaped three-dimensional structure of the lipocalins, which forms a central hydrophobic binding pocket for the ligand, is also characteristic of the fatty acid-binding proteins; these represent another recently identified protein family that also binds small hydrophobic molecules, but which by contrast, contain a 10-stranded antiparallel β-barrel and are almost exclusively intracellular. Because of their similarities of structure and function, Flower and co-workers (1) have proposed the classification of lipocalins and fatty acid-binding proteins into a larger structural superfamily termed calycins.

Although lipocalins are generally soluble proteins, apolipoprotein D (apoD)[1] was originally identified as a component of the plasma high density lipoprotein (HDL) particle, leading to the suggestion that apoD may transport a component of the lecithin-cholesterol acyltransferase reaction (7). Unlike the classical apolipoproteins, which are embedded in the lipoprotein surface by extended amphipathic α-helical structures, homology modeling of apoD against the atomic coordinates of bilin binding protein suggested that apoD associates with the HDL particle by a hydrophobic surface loop. This modeling study also postulated that a heme-related compound may be the preferred ligand for apoD (8). However, apoD has also been identified as a progesterone- and pregnenolone-binding protein isolated from breast fluid, suggesting a role in the transport of steroid hormones in human mammary tissue (9). In the cyst fluid of women with gross cystic disease of the breast, apoD can exceed the concentration found in plasma by about 1000-fold (10), and apoD induction by both retinoic acid (11) and interleukin-1-α (12) has been demonstrated in human breast cancer cells, suggesting that apoD may be a marker of hormonal alterations. Additionally, apoD accumulates in regenerating and remyelinating peripheral nerve, suggesting a role in lipid transport within extravascular compartments (13, 14). Like other members of the lipocalin superfamily, apoD appears to be able to transport a variety of ligands in a number of different contexts.

Despite the presence of lipocalins in a wide range of eukaryotic organisms, no lipocalin has ever been identified in bacteria (15). Additionally, the apolipoprotein components of plasma

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) U21726 and U21727.

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[1] The abbreviations used are: apoD, apolipoprotein D; HDL, high density lipoprotein; Blc, bacterial lipocalin; LB, Luria-Bertani medium; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; IPTG, isopropyl-1-thio-β-D-galactopyranoside.

lipoproteins are unrelated to bacterial lipoproteins, which are anchored to membranes by a lipid-modified amino-terminal cysteine residue (16). In this report, we describe an outer membrane lipoprotein of *Escherichia coli*, which is clearly homologous to apoD. This protein, which we term Blc (bacterial lipocalin), is encoded by the *blc* gene at 94.5 min on the *E. coli* chromosome, immediately downstream of the *ampC* β-lactamase operon. The *blc* promoter is expressed at the onset of stationary growth phase under control of the *rpoS* sigma factor gene, which directs expression of genes necessary for adaptation to starvation conditions. Blc is the first lipocalin identified in a bacterial species and may provide an evolutionary link between bacterial and plasma lipoproteins.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions—E. coli MC4100 (F⁻, Δ*[argF-lac]U169, araD139, rpsL150, ptsF25, flbB5301, rbsR, deoC, relA1*) was provided by G. Cecchini (17), and RH90 (MC4100, *rpoS359*::Tn*10*) was provided by R. Hengge-Aronis (18). *E. coli* TG1 *supE, hsdΔ5, thi, Δ(lac-proAB)*, F′[*traD36, proAB*⁺, *lacI*^{q}, *lacZΔM15*] was obtained from Amersham Corp. Bacteria were grown aerobically in a New Brunswick shaker at 37 °C in Luria Bertani (LB) medium (19). Strains harboring plasmids or transposons were supplemented appropriately with antibiotics (ampicillin at 100 μg/ml, chloramphenicol at 42.5 μg/ml, and tetracycline at 12.5 μg/ml). Overnight cultures were subcultured at a 1% inoculum and growth monitored with a Klett-Summerson colorimeter.

Materials—DNA polymerase from *Thermus aquaticus* and restriction enzymes were obtained from Life Technologies, Inc. Reverse transcriptase from avian myeloblastosis virus, RNase A, bacteriophage T4 polynucleotide kinase, and glycogen were obtained from Boehringer Mannheim, whereas RNAguard was obtained from Pharmacia Biotech Inc. [9,10-^{3}H]Palmitate (54 Ci/mmol) was obtained from Amersham Buchler, whereas [γ-^{32}P]ATP (6 000 Ci/mmol) was obtained from DuPont. Globomycin was a gift from Sankyo Co., Ltd., Tokyo. 4-Methylumbelliferone and 4-methylumbelliferyl-β-D-galactoside were obtained from Sigma. All other materials were obtained from commercial sources.

DNA and Protein Analysis—Plasmid purifications, restriction enzyme digestions, ligations, transformations, and DNA electrophoresis were performed according to Sambrook *et al.* (20). Electroporation was performed with a Cell-Porator (Life Technologies, Inc.) according to the manufacturers instructions. DNA synthesis and sequencing was performed in the Core Facility of the Department of Biochemistry, University of Alberta using an Applied Biosystems model 392 DNA/RNA synthesizer and model 373A DNA sequencer (Perkin Elmer). Protein was determined according to Lowry (21), and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Bishop and Weiner (22).

DNA Sequencing of the blc Gene from E. coli—A 6.1-kilobase *Eco*RI fragment, corresponding to the 94.5 min region of the chromosome from *E. coli* strain CS520, was subcloned from plasmid pLC16−43 (23) into pACYC184 (19). The resulting plasmid (pAmpAC) was propagated in *E. coli* MC4100 and used as a double-stranded template for sequencing both strands of the genomic region between *ampC* (24) and *sugE* (25). The forward primer (EcPst: 5′-GTCGTTGCCTGCAGTTCTCC-3′) hybridizes between nucleotides 148 and 167 (Fig. 1), whereas the reverse primer (EcSma: 5′-AACTACCAGGCTGC TGTACC-3′) hybridizes between nucleotides 615 and 634 (Fig. 1).

Transcriptional Mapping of the E. coli blc Promoter—RNA was prepared by the modified hot phenol extraction method of Frost *et al.* (26) and resuspended in diethylpyrocarbonate-treated water at 10 mg/ml. The primer used in primer extension analysis (EcBlc: 5′-GGAGAACTGCAGGCAACGACC-3′) hybridizes between nucleotides 147 and 167 (Fig. 1). Total cellular RNA (20 μg) was annealed to 0.5 pmol of primer, labeled at the 5′ end with [γ-^{32}P]ATP using T4 polynucleotide kinase (19). The primer and RNA were mixed in a 30-μl volume of annealing buffer (3 M NaCl, 0.5 M Tris-HCl, pH 7.5, and 1 mM EDTA, pH 8.0), denatured at 85 °C for 5 min, and allowed to anneal at 37 °C for a minimum of 1 h. Following annealing, the nucleic acids were precipitated with ethanol and the pellets allowed to air dry before they were resuspended in a 25-μl volume of reverse transcriptase buffer containing 0.5 mM dNTPs and 15 units of RNAguard. Reverse transcriptase (20 units) was added, and the reaction was incubated at 42 °C for 1 h. RNA was removed by treatment with RNase A for 15 min at 37 °C, and the DNA was precipitated with ethanol in the presence of 0.3 M sodium acetate, pH 4.8, and 10 μg of glycogen. The products were separated on a 6% sequencing gel alongside dideoxy sequencing reactions using the same primer as was used for the extension reaction. Gels were dried, and autoradiography was carried out at −70 °C for 3 weeks.

Construction and Analysis of a blc::lacZ Translational Fusion Plasmid—A 4-kilobase *Eco*RI/*Kpn*I fragment from pAmpAC was cloned into pLacZY2 (27) to create a translational fusion plasmid (pBlcLacEK) in which the first 42 codons of *blc* are followed by *lacZ*. A 1.8-kilobase *Bam*HI/*Cla*I fragment from pMF5 (28) was cloned into pACYC184 to create pACKatF, which carries a functional allele of *rpoS* on a vector that is compatible with pLacZY2 and pBlcLacEK; these latter two plasmids could only be transformed into *E. coli* MC4100 and RH90 by electroporation. β-Galactosidase activity was monitored fluorometrically using 4-methylumbelliferyl-β-D-galactoside as substrate according to the method of Youngman (29), except that activities were normalized against total protein assayed (in milligrams) rather than the optical densities of the cultures. Measurements were determined with a Sequoia-Turner model 450 fluorometer equipped with NB360 and SC430 filters.

Construction of an Inducible Blc Expression Plasmid—Amplification of *blc* from *E. coli* by the polymerase chain reaction (PCR) was performed according to Bishop and Weiner (22) using the forward primer (EcEco: 5′-ATATGAATTCAAGAGGAAACATTTATGCGCC-3′) for which the 18 residues at the 3′ end hybridize between nucleotides 92 and 109 (Fig. 1); the 17 residues at the 3′ end of the reverse primer (EcHind: 5′-TATAAAGCTTTAACTACCAGGCTGCTG-3′) hybridize between nucleotides 619 and 635 (Fig. 1). PCR was performed through 25 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 30 s, using pAmpAC as template. The PCR product was digested with *Eco*RI and *Hin*dIII for cloning into expression vectors pMS119EH and pMS119HE (30) digested with the same restriction enzymes; the resulting plasmids were designated pBlcEH and pBlcHE, respectively, and differ only in the orientation of the *blc* gene with respect to the inducible *tac* promoter. The *blc* gene cloned behind the *tac* promoter (pBlcEH) was subjected to double-stranded sequencing as indicated above using the same primers for the PCR amplification.

Membrane Isolation and Fractionation—E. coli MC4100 (250 ml) harboring pBlcEH or pBlcHE was grown for 2 h and induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) for an additional 2 h before harvesting. Cells were resuspended in 10 ml of ice-cold Buffer A (50 mM sodium phosphate, pH 7.1, 10 mM $MgCl_2$), adjusted to 10 μg/ml in DNase, and mechanically disrupted by passage through a precooled French press cell at 1000 p.s.i. Unbroken cells were removed by low speed centrifugation at 4 °C before the membranes were sedimented at 90,000 rpm in a Beckman TL 100.3 rotor for 30 min. Samples of the supernatant and membrane fractions, as well as the whole cell lysates, were analyzed by SDS-PAGE. The membranes were resuspended in 200 μl of ice-cold Buffer A and layered on top of a discontinuous sucrose gradient composed of 0.5 ml of 60%, 1 ml of 55%, 2.4 ml of 50%, 2.5 ml of 45%, 2.4 ml of 40%, 1.4 ml of 35%, and 0.8 ml of 30% sucrose in Buffer A. Centrifugation was at 4 °C for 16 h at 35,000 × *g* in a Beckman SW41 rotor (31). The separated membranes were harvested from the centrifuge tube and analyzed by SDS-PAGE; sucrose was removed by trichloroacetate precipitation as described by Bishop and Weiner (22).

Palmitate Labeling of Induced Protein—For palmitate labeling, 25-ml cultures of *E. coli* MC4100 harboring pBlcEH or pBlcHE were grown for 1 h, and 10 ml were transferred into growth flasks containing 50 μCi of [^{3}H]palmitate from which toluene had been evaporated over a gentle stream of N_2(g). The cultures were made 1 mM in IPTG, 100 μg/ml in ampicillin and, when necessary, 10 μg/ml in globomycin. Cells were grown an additional 1 h, harvested, washed once with 50 mM sodium phosphate, pH 7.2, and resuspended in 120 μl of 20 mM Tris-HCl, pH 8, containing 1 mM EDTA and 1% SDS. Cells were incubated for 5 min at 100 °C and centrifuged at 14,000 × *g* for 10 min. The supernatant (100 μl) was added to 1 ml of ice-cold acetone and kept overnight at 4 °C before centrifugation as above. The pellet was thoroughly resuspended in 50 μl of 1% SDS, and an aliquot corresponding to about 800,000 cpm was separated by 15% SDS-PAGE and the labeled protein visualized by fluorography. Relative molecular mass was estimated using Rainbow low molecular mass standards (Amersham Corp.).

Computer Methods—Sequence alignments were performed using the GCG (Genetics Computer Group, University of Wisconsin) FASTA and TFASTA algorithms. Protein structure was analyzed using FRODO (32) and MOLSCRIPT (33).

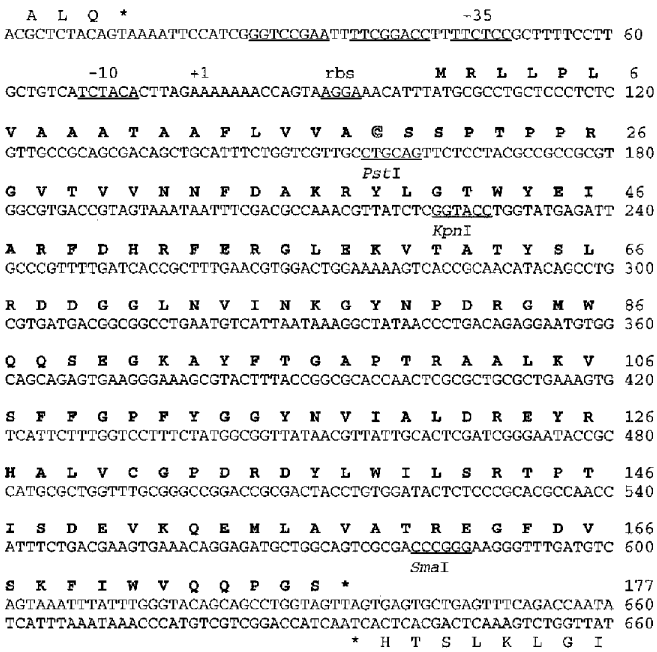

FIG. 1. **Nucleotide sequence of the *blc* gene from *E. coli* CS520.** The sequence of the *blc* gene from pAmpAC extends over 660 base pairs beginning with the final four codons of the *ampC* gene and its rho-independent terminator (*underlined*), followed by the −35 and −10 regions of the *blc* promoter, the *blc* transcriptional start (+1) and ribosomal binding (*rbs*) sites, through the 177 amino acid residues of the Blc open reading frame (*bold type*), and ending with the final 9 codons of the convergent *sugE* gene. The *Pst*I, *Kpn*I, and *Sma*I restriction enzyme sites are *underlined*, and the cysteine residue at the lipoprotein cleavage site is *outlined*.

RESULTS

Sequence Analysis of the blc Gene—Approximately 370 base pairs of DNA that remained to be characterized between the 3′ end of *ampC* and the 3′ end of *sugE*, which is oriented toward *ampC* in the 94.5 min region of the *E. coli* chromosome, was subjected to DNA sequencing. From the published sequence of *ampC* (24), we designed a forward sequencing primer (EcPst; see "Experimental Procedures"), whereas the published sequence of *sugE* (25) allowed us to design a reverse sequencing primer (EcSma; see "Experimental Procedures"). By utilizing the genomic DNA from *E. coli* CS520 carried on plasmid pAmpAC as a double-stranded template, it was possible to unambiguously assign the sequence of both DNA strands between *ampC* and *sugE*. The 660 base pairs that separate *ampC* and *sugE* in *E. coli* CS520 are shown in Fig. 1.

A single open reading frame was revealed, which specified 177 amino acid residues (19,853 Da) and exhibited a consensus prokaryotic lipoprotein cleavage site (16) predicting a mature protein of 159 residues (18,043 Da). The initiating methionine codon was separated from the rhoindependent terminator of the *ampC* gene by 60 base pairs, from which −35 and −10 hexamers corresponding weakly to an *E. coli* σ-70 promoter could be distinguished. A reasonable ribosome binding sequence was appropriately positioned upstream of the initiating methionine codon. The open reading frame converged upon *sugE* such that the two genes shared overlapping translational termination codons; no rhoindependent terminator structure could be discerned between them. A chromosomal deletion in *E. coli* MI1443 encompasses the *blc* locus; this strain is capable of growth under both aerobic (34) and anaerobic (35) conditions, indicating that the *blc* gene is dispensable in *E. coli*.

While this manuscript was in preparation, the DNA sequence of the *E. coli* MG1655 chromosomal region from 92.8–0.1 min was submitted from the *E. coli* genome project under GenBank accession number U14003. The sequence from *E. coli* MG1655 confirms that determined by us for *E. coli* CS520, except for a C to G transversion at nucleotide 498; this is a silent mutation in the third position of glycine codon 132. We also determined the sequence of the *blc* homolog from *Citrobacter freundii* OS60; this DNA sequence, together with neighboring loci, will be described elsewhere. Pertinent to this study was the identification of an open reading frame specifying 177 amino acid residues and displaying 90% amino acid sequence identity with *E. coli* Blc.

Transcriptional Mapping of the E. coli blc Promoter—To verify that the *blc* gene was expressed, primer extension analysis was performed using the primer EcBlc (see "Experimental Procedures"). A weakly expressed cDNA corresponding to a transcript whose 5′ end mapped to nucleotide 80 in Fig. 1 was detected from *E. coli* MC4100 (Fig. 2). Autoradiography was performed for 3 weeks in order to detect this cDNA. The expression of the *blc* mRNA was improved if the cells were allowed to grow into stationary phase.

E. coli MC4100 was recently reported to be distinct from many other common strains of *E. coli* by virtue of having a functional allele of *rpoS*, which controls a program of gene expression induced under starvation conditions and at the onset of stationary phase (36, 37). In order to determine if the accumulation of the *blc* transcript in stationary phase was determined by *rpoS*, we also performed primer extension analysis using *E. coli* RH90 (MC4100 *rpoS359*::Tn*10*). The *blc* transcript was not detected in *E. coli* RH90 under all observed growth phases (Fig. 2), indicating that the *blc* gene belongs to the stationary phase regulon controlled by *rpoS*, and suggesting that the Blc protein may serve a function that contributes to the adaptation of cells to starvation conditions. Although there is no clearly defined consensus sequence for *rpoS*-dependent promoters, they are generally similar in structure to those controlled by σ-70 (38, 39).

Monitoring Activity of a blc::lacZ Translational Fusion in Vivo—To determine if the primer extension results were valid *in vivo*, a *blc::lacZ* translational fusion was created from pLacZY2, which was developed to exhibit low background β-galactosidase activity, thus permitting the analysis of weakly expressed genes (27). The results (Table I) demonstrate that activities expressed from the fusion plasmid (pBlcLacEK) were only significantly above those expressed from pLacZY2 when a functional *rpoS* allele was present, indicating that σ-70 has no detectable effect on the *blc* promoter. A slight 1.5-fold increase above background activity was determined in exponential phase cultures (200 min of growth) in the presence of the chromosomal *rpoS* gene from *E. coli* MC4100, and this was matched by a 1.4-fold increase in the presence of the plasmid encoded *rpoS* gene (pACKatF) harbored by *E. coli* RH90. However, in stationary phase cultures (overnight growth), the chromosomal *rpoS* gene provided a 2.5-fold increase above background activity, whereas the plasmid encoded *rpoS* gene provided a 5.3-fold increase. These results confirm that *blc* is normally expressed only very weakly in *E. coli* and that this expression is optimized in stationary phase under the control of *rpoS*.

Controlled Expression of Blc Protein—To express the Blc protein, the *blc* gene from *E. coli* was cloned behind the IPTG-inducible *tac* promoter in the expression vector pMS119EH to create pBlcEH. The *blc* gene was also cloned in the reverse orientation in pMS119HE to create pBlcHE, which serves as a negative control. The gene was amplified by PCR using primers designed to incorporate an *Eco*RI restriction enzyme site and improved ribosome binding sequence at the 5′ end (EcEco; see "Experimental Procedures") and a *Hin*dIII restriction enzyme

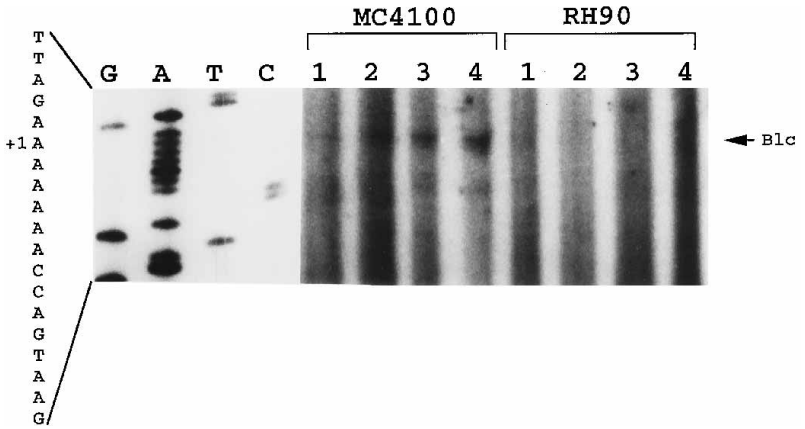

FIG. 2. **Transcriptional mapping and *rpoS* control of the *blc* promoter.** The deduced sequence of the *blc* promoter region derived from the sequencing lanes labeled *G*, *A*, *T*, and *C* is shown *vertically* on the *left*. The primer extension reactions were performed with total cellular RNA isolated from *E. coli* strains MC4100 (*rpoS*$^+$) and RH90 (MC4100, *rpoS359*::Tn*10*). RNA samples were isolated at mid-exponential (*1*), late exponential (*2*), early stationary (*3*), and stationary (*4*) growth phases, which were reached after 200, 270, 340, and 540 min of growth, respectively. The position of the cDNA corresponding to the *blc* transcription start site (+1) is marked by an *arrowhead*.

TABLE I
β-Galactosidase activities expressed from a blc::lacZ translational fusion plasmid

β-Galactosidase specific activities were monitored in exponential phase and stationary phase cultures from a chimeric *blc::lacZ* translational fusion plasmid (pBlcLacEK) and the promoterless control plasmid (pLacZY2) in the presence and absence of a functional *rpoS* allele, which was carried either on pACKatF or on the chromosome of *E. coli* MC4100. Results are the mean values (± standard deviation) of three independent fluorometric assays using 4-methylumbelliferyl-β-D-galactoside as substrate.

Strain	Plasmid	β-Galactosidase specific activity	
		Exponential growth phase	Stationary growth phase
		pmol/min·mg	
MC4100 (*rpoS*$^+$)	pLacZY2	13 ± 1	13 ± 2
MC4100 (*rpoS*$^+$)	pBlcLacEK	19 ± 1	33 ± 2
RH90 (*rpoS*$^-$)	pLacZY2	15 ± 1	14 ± 2
RH90 (*rpoS*$^-$)	pBlcLacEK	16 ± 1	16 ± 1
RH90 (*rpoS*$^-$)	pACKatF (*rpoS*$^+$) pLacZY2	16 ± 3	15 ± 1
RH90 (*rpoS*$^-$)	pACKatF (*rpoS*$^+$) pBlcLacEK	23 ± 2	80 ± 10

site at the 3′ end (EcHind; see "Experimental Procedures"). The DNA sequence of the PCR product in pBlcEH was identical to that of the template, except for a G to A transition at nucleotide 516; this is a silent mutation in the third position of leucine codon 138. Blc was induced in *E. coli* MC4100 and protein divided into cellular lysate, soluble, and membrane fractions. Samples analyzed by SDS-PAGE are shown in Fig. 3. A doublet of bands migrating with molecular masses of 16 and 18 kDa in the Blc expressing lanes were visible in the cellular lysate and the membrane fractions. This finding is consistent with an accumulation of a membrane protein, which is being processed to a mature species by the removal of an 18-amino acid signal peptide and the addition of 700 Da, the approximate molecular mass attributed to lipid modification of prokaryotic lipoproteins. The two unique bands in the Blc expressing lanes migrate slightly faster than their expected positions of 18 and 20 kDa for the mature and precursor species of Blc, respectively. This anomolous migration may result from lipid modification of Blc, which would promote binding of SDS and facilitate migration in SDS-PAGE.

Subcellular Localization of Blc Protein—To localize the two Blc species in the Gram-negative cell envelope of *E. coli*, membranes were fractionated by sucrose density gradient centrifugation. Light, heavy, and medium fractions were harvested; these represent the cytoplasmic membrane, outer membrane, and adhesion zones between outer and cytoplasmic membranes, respectively (31). The band corresponding to the Blc precursor was localized in the cytoplasmic membrane fraction, whereas the band corresponding to mature Blc was localized in the outer membrane fraction, and both species were equally distributed among the fraction of adhesion zones (Fig. 4). This result suggests that the Blc precursor accumulates in the cytoplasmic membrane, and that mature Blc is targeted to the outer membrane.

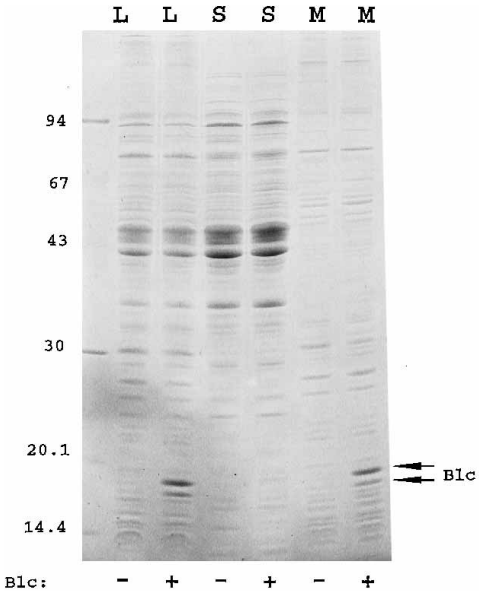

FIG. 3. **Overexpression and membrane localization of the Blc protein.** 12% SDS-PAGE analysis of *E. coli* MC4100 transformed with either pBlcEH, expressing *blc* (+), or pBlcHE, unable to express *blc* (−). Cells were induced with IPTG and subjected to French pressure lysis. The lysates (*L*) were divided into soluble (*S*) and membrane (*M*) fractions by ultracentrifugation. The position of bands present during Blc expression are marked by *arrowheads*. Each lane corresponds to 40 μg of protein stained with Coomassie Blue dye. The indicated molecular mass standards are expressed in kDa.

Palmitate Labeling of the Blc Protein—Further evidence of the lipoprotein nature of the Blc protein was obtained by ex-

pressing the protein in the presence of [^{3}H]palmitate. A major band near 16 kDa was detected by fluorography after SDS-PAGE only when Blc was expressed (Fig. 5). Addition of globomycin (40), an inhibitor of the lipoprotein signal peptidase (signal peptidase II), inhibited the formation of the 16-kDa band concomitantly with the formation of an 18-kDa band (Fig. 5); this latter species likely represents the diacylglyceryl-prolipoprotein form of Blc. The heavy band near 5 kDa corresponds to the murein lipoprotein for which a larger diacylglyceryl-prolipoprotein form also appears in the presence of globomycin (16).

Homologies between Blc and ApoD—A sequence alignment using TFASTA (Genetics Computer Group) revealed that the *E. coli* and *C. freundii* Blc species exhibited homology with Vlp, an uncharacterized open reading frame derived from *Vibrio cholerae* (Table II). Additionally, the bacterial proteins scored between 26 and 33% amino acid sequence identity over their entire length with three species of mammalian apoD. Lower level similarities were detected with a number of other lipocalins. The initial alignments were optimized by comparison of the primary structure of Blc with the tertiary structure of human apoD using FRODO (32). The resulting alignment is shown in Fig. 6.

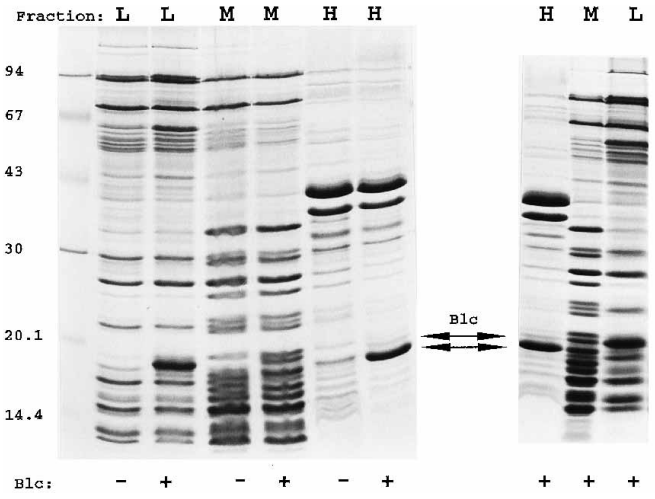

FIG. 4. **Membrane fractionation of precursor and mature species of Blc.** 15% SDS-PAGE analysis of *E. coli* MC4100 transformed with either pBlcEH, expressing *blc* (+), or pBlcHE, unable to express *blc* (−). Cells were induced with IPTG and subjected to French pressure lysis. Membranes were isolated by ultracentrifugation and separated into light (*L*), medium (*M*), and heavy (*H*) fractions by sucrose density gradient centrifugation. The pBlcEH fractions shown at *right* are aligned to show the precursor and mature forms of Blc. The position of bands present during Blc expression are marked by *arrowheads*. Each lane corresponds to 40 μg of protein stained with Coomassie Blue dye. The indicated molecular mass standards are expressed in kDa.

DISCUSSION

We have identified a novel bacterial lipoprotein (Blc), which exhibits homology with a eukaryotic lipocalin (apoD). Blc is optimally expressed in stationary phase and is under the control of the RpoS σ-factor global regulator. In the natural environment, bacteria spend the majority of their existence in the stationary phase and the expression of genes during stationary phase is of considerable interest. Bacteria have developed sophisticated mechanisms to survive starvation for prolonged periods of time, and it has been proposed that proteins synthesized at the late stages of growth are important for the survival of the organism (41, 42). It is now apparent that several *E. coli* lipoproteins are expressed under stationary phase conditions. A starvation-inducible lipoprotein (Slp) was recently shown to be expressed in stationary phase cultures independently of *rpoS* (43), whereas stationary phase expression of the OsmB lipoprotein was shown to require *rpoS* (44). The *rpoS*-dependent expression of the *blc* gene suggests that Blc, like the other stationary phase lipoproteins, may serve an important starvation response function in *E. coli*.

A Blc-like protein has been found in *E. coli*, *C. freundii*, and *V. cholerae* and it presumably exists in other *Enterobacteriaceae*. Although an *E. coli* strain deleted for the *blc* gene grows normally in the laboratory, *blc* may offer some advantage in the pathogenic or natural environment. Based on the hydrophobic ligand binding capacity of apoD, it seems likely that Blc also functions to bind a hydrophobic ligand. The Blc mRNA is very

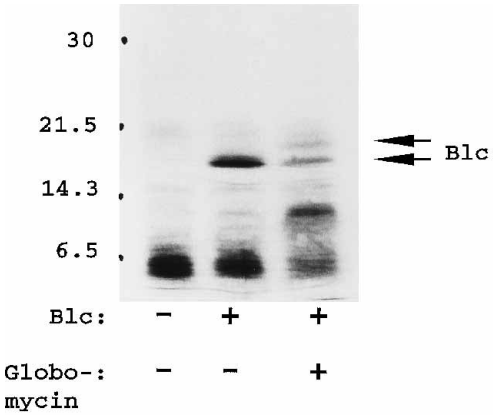

FIG. 5. **Metabolic labeling of Blc with [^{3}H]palmitate.** 15% SDS-PAGE analysis of *E. coli* MC4100 transformed with either pBlcEH, expressing *blc* (+), or pBlcHE, unable to express *blc* (−). Cells were induced with IPTG in the presence of [^{3}H]palmitate, and globomycin was added when indicated (+). Proteins were prepared by boiling the cells in SDS, and samples corresponding to 800,000 cpm were resolved by 15% SDS-PAGE and visualized by fluorography. The position of bands present during Blc expression are marked by *arrowheads*. The indicated molecular mass standards are expressed in kDa.

TABLE II

Comparison of amino acid sequence identities between apolipoprotein D and bacterial lipocalin species

The primary structures of Blc from *E. coli* (EcBlc; this paper) and *C. freundii* (CfBlc; this paper) are compared with Vlp from *V. cholerae* (VcVlp; GenBank accession no. X64097), apoD from *Mus musculus* (MmApd; GenBank accession no. X82648), apoD from *Rattus norvegicus* (RnApd; GenBank accession no. X55572), and apoD from *Homo sapiens* (HsApd; GenBank accession no. J02611). The values represent percentage identities defined as number of identical residues between each pair of mature polypeptides as aligned in Fig. 6, divided by the number of residues in the shortest member of the pair.

	VcVlp	EcBlc	CfBlc	MmApd	RnApd	HsApd
VcVlp	100	49	48	26	28	26
EcBlc		100	90	33	33	31
CfBlc			100	32	33	31
MmApd				100	89	74
RnApd					100	73
HsApd						100

```
VcVlp   ---------MRAIFLILCSVLLNGCLGMPESVKPVSDPELNNYTGKWYEVA   42
EcBlc   ---MRLLPLVATATAAFLVVACSSPTPPRGVTVVNDFDAKRYTGTWYEIA   47
CfBlc   ---MRILPVVAAVTAAFLVVACSSPTPPKGVTVVNDFDAKRYTGTWYEIA   47
MmApd   MVTMLMFLATLAGLFTTAKGQNFHLGKCPSPPVQENLDVKKYLGRWYEIE   50
RnApd   MATMLLLLATLAGLFTTTEGQSFHLGKCPSPPVQENFDVKKYLGRWYEIE   50
HsApd   MVMLLLLLSALAGLFGAAEGQAFHLGKCPNPPVQENFDVNKYLGRWYEIE   50
                                                  bbbbbbb

VcVlp   RLDHSFFRGLSQVTATFTRVRNLGGISVLNRGYSEEKGEWKEATGKAYFVN   92
EcBlc   RFDHRFFRGLEKVTATYSLRDLGGLNVINKGYNPDRGMWQQSEGKAYFTG   97
CfBlc   RFDHRFFRGLDKVTATYSLRDLGGTNVINKGYNPDREMWQKTEGKAYFTG   97
MmApd   KIPASFFKG-NCIQANYSLMENGNLEVLNKELSPDG-TMNQVKGEAKQSN   98
RnApd   KIPVSFFKG-NCIQANYSLMENGNLKVLNKELRPDG-TLNQVEGEAKQSN   98
HsApd   KIPTTFFNG-RCIQANYSLMENGKLKVLNQELRADG-TVNQIEGEATPVN   98
        b         bbbbbbbbb       bbbbbbb       bbbbbbbbb

VcVlp   GSTDGYLKYSTDGPF-YGSTVVFEIDRTNYSYALVSGP-------NTEYL   134
EcBlc   APTRTAALKVSTDGPF-YGGYNVIALDRE-YRHALVCGP-------DREYL   138
CfBlc   DPSTAALKVSTDGPF-YGGYNVIALDRE-YRHALVCGP-------DREYL   138
MmApd   VSEPAKLEVQFFPLMPPAPYWILATDYE--NYALVYSCTTFFWLFHVDFF   147
RnApd   MSEPAKLEVQFFSLMPPAPYWILATDYE--SYALVYSCTTFFWFFHVDYV   147
HsApd   LTEPAKLEVKFSWFMPSAPYWILATDYE--NYALVYSCTCIIQLFHVDFA   147
           bbbbbbb       bbbbbbbbbb     bbbbbbbbb     bbbbbbb

VcVlp   WLLSRTPTVERGILDKFIEMSKERGFDTNRLIYVQLQ------          171
EcBlc   WLLSRTPTISDEVKQEMLAVATREGFDVSKFIWVQQPGS----          177
CfBlc   WLLSRTPTISDEMKQQMLAIATREGFEVNKLIWVKQPGA----          177
MmApd   WILGRNPYLPPETITYLKDILTSNGIDIEKMTTTDQANCPDFL          189
RnApd   WILGRNPYLPPETITYLKYILTSNDIDIAKITTKDQANCPDFL          189
HsApd   WILARNPNLPPETVDSLKNILTSNNIDVKTMTVTDQVNCPKLS          189
        bbb       hhhhhhhhhhh
```

FIG. 6. **Homologies between apolipoprotein D and bacterial lipocalins.** The bacterial lipocalins are aligned with three apoD species; see Table II for the source of each sequence. Residues are *shaded* when at least 5 of the 6 aligned residues are identical. The positions of cysteine residues forming the two disulfide bonds in the apoD species are *underlined* in *bold type* and one pair distinguished by *italics*. Additional cysteine residues are shown in *bold type* and include the positions of the lipoprotein cleavage site near the amino terminus of the Vlp and Blc species; the amino-terminal glutamine residues of the mature apoD species are also shown in *bold type*. The homologies were initially identified using the TFASTA algorithm (Genetics Computer Group) and optimized manually by comparison with the secondary structural elements of human apoD, which are shown under the alignment as β-strands (*b*) and α-helix (*h*).

poorly expressed, suggesting that the normal level of Blc in the outer membrane is low and that Blc is not simply sequestering hydrophobic ligands. Most outer membrane lipoproteins are oriented toward the periplasm and we presume that Blc serves to capture its hydrophobic ligand within the periplasm, although we have not eliminated the possibility that the protein serves as an external receptor molecule. Studies are in progress to characterize the orientation of Blc.

The lipocalin proteins are composed of a common structural motif that consists of an eight-stranded antiparallel β-barrel, arranged as two stacked orthogonal sheets, with a COOH-terminal α-helix. A large proportion of lipocalin residues are exposed to the hydrophobic interior and reside in the central ligand binding cavity. It has been suggested that different lipocalins change these residues to accommodate different ligand specificities, thus explaining the rather low levels of primary structural homology despite the common lipocalin fold (6). In this context, the 26–33% amino acid sequence identity that exists between the three species of apoD and the three bacterial lipocalins must be regarded as highly significant (Table II and Fig. 6). Molecular modeling of apoD against the atomic coordinates of bilin-binding protein (8), which together share only 27% amino acid sequence identity, predicted a disulfide bonding pattern in apoD that has since been confirmed biochemically (45). Additionally, the apoD model led to the identification of a hydrophobic surface loop between β-strands 7 and 8, which was postulated to mediate the physical interaction between apoD and HDL (Fig. 7). It has since been shown that a lone cysteine residue located immediately adjacent to the hydrophobic loop in human apoD forms an intermolecular disulfide bond with the HDL-associated apolipoprotein AII, providing strong support for the proposed interaction (45). Interestingly, the aforementioned cysteine residue is absent in the rodent apoD species (Fig. 6), which is consistent with the observation that rat apoD is not found to a significant extent on plasma lipoproteins (13).

A 7-amino acid deletion in the bacterial lipocalins appears to have eliminated the corresponding hydrophobic surface loop found in the apoD species (Fig. 6). Additionally, a glycine and a proline residue are suitably positioned at the leading edge of the deleted loop to accommodate a β-hairpin turn. A lone cysteine preceding this putative turn structure is evident in the *E. coli* and *C. freundii* Blc proteins, but not in the *V. cholerae* Blc, potentially implicating a species-specific disulfide between Blc and itself or another component of the bacterial cell envelope. Two intramolecular disulfide bonds in the apoD species are formed by four additional cysteine residues, which are absent in the three bacterial lipocalins. The only other cysteine residue in the Blc proteins is located at the lipoprotein processing site near the amino terminus. The *in vivo* localization of *E. coli* Blc in the outer membrane is consistent with the presence of a serine residue at the +2 position of the mature protein (46).

As Blc represents the first lipocalin to be characterized from a bacterium, it raises important questions regarding the origin of this burgeoning class of proteins. Blc is unique among bac-

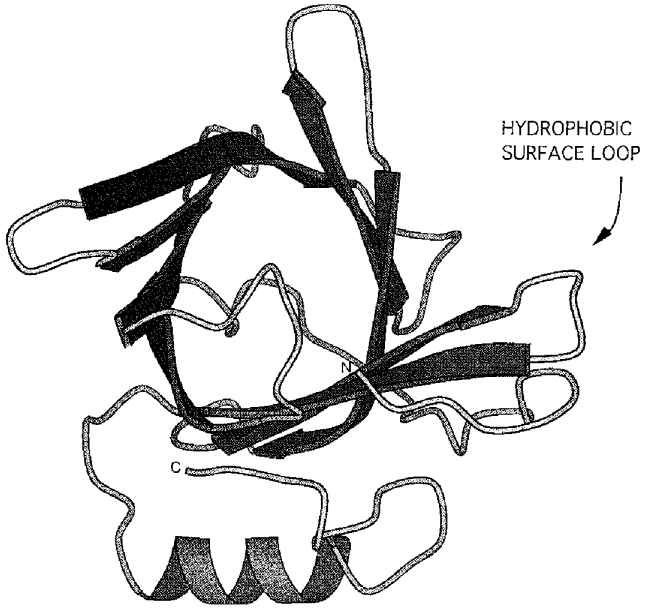

FIG. 7. **Molecular model of human apolipoprotein D.** Molecular model of human apoD emphasizing the eight antiparallel β-strands, which form two orthogonal sheets, followed by a COOH-terminal α-helix. The hydrophobic surface loop that separates β-strands 7 and 8 is marked with an *arrowhead*. This MOLSCRIPT diagram was generated by Natalie Strynadka using the coordinates of human apoD obtained from the Brookhaven Protein Data Bank under accession number 2APD.

terial lipoproteins in that it is a lipocalin, whereas apoD is unique among lipocalins in that it is a plasma lipoprotein. Did the primordial lipocalin originate in bacteria, where it served a function in the Gram-negative cell envelope before it was acquired by eukaryotes and adapted to a number of functions in multicellular organisms, or did the lipocalins originate firstly in eukaryotes from where they were adapted, at least in the case of apoD, to function in the Gram-negative cell envelope? What should be clear from this study is that the function of apoD either originates from or has been adapted toward a basic function in the cell envelope of *E. coli*. In this regard, it is remarkable that Blc and apoD are both found anchored in asymmetric bilayers; the inner leaflet of the Gram-negative outer membrane and the plasma exposed surface of mammalian lipoproteins are two rare examples of non-phospholipid bilayer membranes in biology. Perhaps the still undefined physiological functions of Blc and apoD may prove to be more closely related than otherwise anticipated. The *rpoS*-dependent activation of the *blc* gene at the onset of stationary phase suggests that the Blc protein may somehow serve in adaptation to starvation conditions. We hope that further analysis of Blc structure and function will be relevant to apoD and its pathology.

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