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Molecular Cloning and Expression in *Escherichia coli* K-12 of the O Antigens of the Inaba and Ogawa Serotypes of the *Vibrio cholerae* O1 Lipopolysaccharides and Their Potential for Vaccine Development

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The gene clusters that determine the biosynthesis of both the Inaba and Ogawa serotypes of the O antigen of the lipopolysaccharide of *Vibrio cholerae* were cloned and expressed in *Escherichia coli* K-12. Restriction analysis of the clones demonstrated that about 15 kilobases were common to all clones and a further 5 kilobases were common to the Ogawa clones. The O antigens expressed by *E. coli* K-12 had the specificity of *V. cholerae*. Antibodies raised against *E. coli* K-12 that harbor one of these clones, pPM1001 (Inaba), were as highly protective in the infant mouse model system as were antibodies to *V. cholerae* itself. Introduction of such clones into suitable carrier strains could be expected to produce a good oral immunogen against cholera.

The outer membrane of gram-negative organisms such as *Vibrio cholerae* contains several major components; among these is the lipopolysaccharide (LPS), which provides a major protective barrier (23, 24). This molecule consists of basically three regions: the lipid A region which is hydrophobic and forms part of the lipid bilayer of the outer membrane; the core oligosaccharide, and the O antigen. The outermost region, the O antigen, provides the major antigenic variability of the cell surface.

In *V. cholerae* strains of the O1 serotype two major subclasses of O antigen are recognized: Ogawa and Inaba. Strains of the Ogawa serotype are said to have the A and B antigens, whereas those of the Inaba serotype are said to have the A and C antigens (3). That is, they both share A, while B is Ogawa specific and C is Inaba specific. However, Ogawa strains may possess small amounts of C antigen (28, 30).

A third, less common serotype, Hikojima, is also found in *V. cholerae* O1 strains (3). Hikojima strains produce antigen A and high levels of both antigens B and C. Bhaskaran (2) found this serotype to be unstable and suggested that Hikojima strains represent segregating merodiploids (cited in reference 25). Alternatively, because it seems possible to get interconversion between the serotypes (8, 29, 30), Hikojima strains could represent strains which undergo conversion at an elevated frequency.

Although the chemical composition of the O antigen has been determined, the actual structure is unknown (12, 26, 27). Of the genetics of O-antigen biosynthesis, all that is known is that variation can be crudely mapped by conjugation to a region called *oag* (25).

It has been demonstrated in model systems that antibodies to two classes of antigens are protective against cholera (21). These are the O antigen of the LPS and certain proteins; antibodies to the protein antigens are more protective on a weight basis. However, the LPS is by far the more potent immunogen. Presumably, antibodies to these components protect by inhibiting adherence and colonization. Little is known about the protective antigens in humans, except that human convalescent sera contain antibodies to both the LPS and a very limited number of proteins (31).

Only in *Salmonella* species have the genes that determine an O antigen been analyzed in any detail, and results of deletion analysis have suggested that these genes, the *rfb* gene cluster, are co-controlled (15, 22). However, neither the O-antigen gene cluster from *Salmonella* species nor any other organism has been cloned and analyzed to determine whether this is so.

In this report we describe the molecular cloning and expression in *Escherichia coli* K-12 of the genes that determine the O antigen of *V. cholerae* of the Inaba form derived from strain 569B and of the Ogawa form derived from strain O17.

MATERIALS AND METHODS

Bacterial strains and media. *V. cholerae* 569B and O17 were from the departmental collection. *E. coli* K-12 DH1 (F^- *gyrA96 recA1 relA1 endA1 thi-1 hsdR17 supE44* λ^-) was obtained from B. Bachmann of the *E. coli* Genetic Stock Center (10). Plasmid pHC79 was obtained from J. Collins (14). *V. cholerae* strains were routinely grown in brain heart infusion broth (Difco Laboratories, Detroit, Mich.), and *E. coli* K-12 derivatives in nutrient broth (Difco) made at double strength with 5 g of NaCl per liter added. Nutrient agar was blood agar base (Difco) without the addition of blood.

Antibiotics were used at the following concentrations: ampicillin, 25 μg/ml; tetracycline, 10 μg/ml.

Chromosomal DNA preparation. Cells were grown overnight with aeration at 37°C in 100 ml of brain heart infusion broth (Difco). The cells were then centrifuged 5,000 × *g* for

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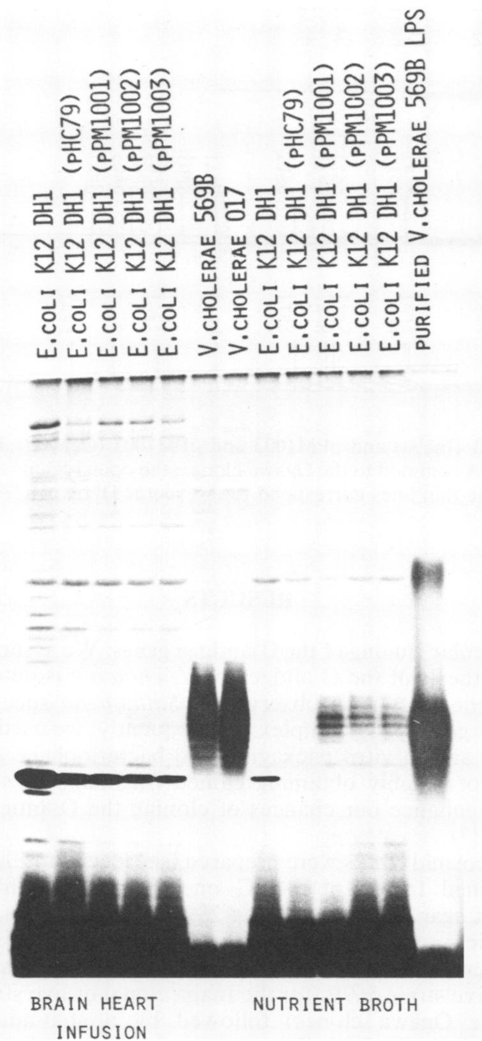

FIG. 1. Cell envelopes of *V. cholerae* 569B and O17 and *E. coli* K-12 DH1 and its derivatives. The *E. coli* K-12 derivatives were grown in either brain heart infusion broth (as were the *V. cholerae* strains) or nutrient broth (Difco). The envelopes prepared by the small-scale method described previously (21) were solubilized in SDS and electrophoresed in SDS on 20% polyacrylamide gels by the method of Lugtenberg et al. (16), as modified previously (1). The gels were fixed so that LPSs or lipoproteins were predominantly stained with silver (34).

10 min at room temperature and washed once in 20 ml of TES buffer (50 mM Tris hydrochloride, 5 mM EDTA, and 50 mM NaCl [pH 8.0]), followed by centrifugation again; and the washed cells were then suspended in 1/50th of the original volume (i.e., 2.0 ml) in cold 25% sucrose in 50 mM Tris hydrochloride (pH 8.0). To this was added 1.0 ml of 5 mg of lysozyme per ml in 0.25 M EDTA (pH 8.0), and the suspension was allowed to stand on ice for 10 min. To this was added 1 ml of 10 mg of pronase per ml in TE buffer (10 mM Tris hydrochloride, 1 mM EDTA [pH 8.0]). This was incubated for 10 min at 37°C followed by the addition of 0.25 ml of lysis solution (5% Sarkosyl [CIBA-GEIGY Corp., Summit, N.J.] in 50 mM Tris hydrochloride, 0.25 M EDTA [pH 8.0]). This was incubated for 60 to 120 min at 37°C with occasional gentle swirling. Lysis became apparent at this time. The lysate was then transferred to a 50-ml Erlenmeyer flask and gently extracted three times with phenol saturated with TE buffer (this was made by adding 1 g of phenol per ml of TE buffer). The residual phenol was then removed by extracting twice with diethyl ether. The DNA solution was then dialyzed overnight against TE buffer at 4°C. The concentration of DNA was usually in the range of 800 to 1,300 μg/ml.

Construction of cosmid gene banks. Genomic fragments of approximately 40 kilobases (kb) were obtained by controlled partial digestion with the restriction endonuclease *Sau*3A. The cosmid vector used was pHC79, and this was restricted with *Bam*HI and treated with molecular biology grade alkaline phosphatase (Boehringer Mannheim Biochemicals, Sydney, Australia) to prevent self-ligation (14). The two DNAs were mixed, ligated overnight, and packaged in vitro into bacteriophage λ. The packaged phage were then used to infect restrictionless (*hsdR*), recombination deficient (*recA*)

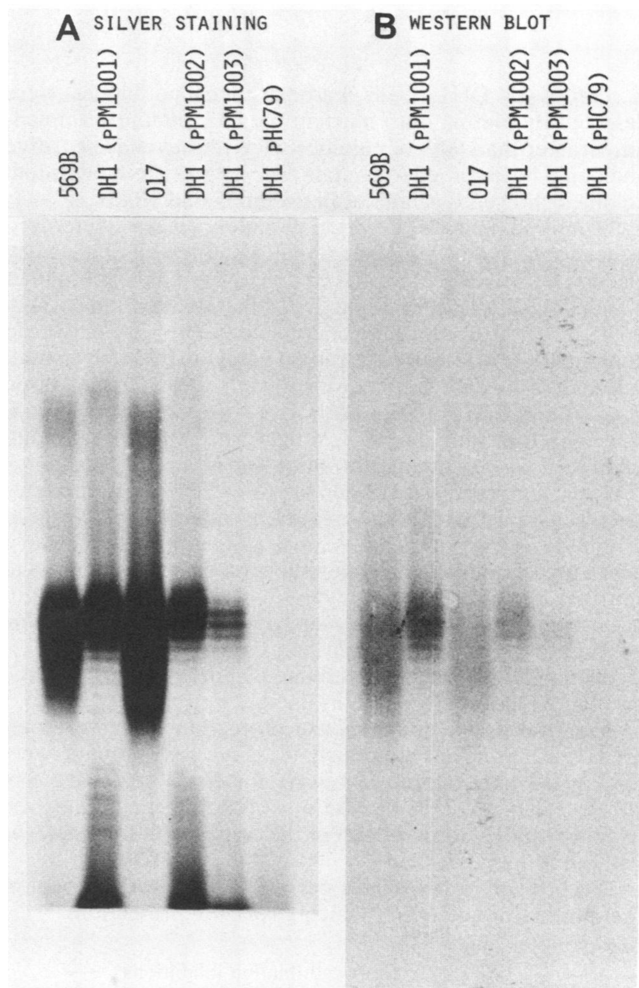

FIG. 2. Comparison by SDS-gel electrophoresis on 20% polyacrylamide of LPSs from *V. cholerae* 569B (classical and Inaba) and O17 (El Tor and Ogawa), and *E. coli* K-12 DH1 harboring either the cosmid vector pHC79 or the O-antigen clones pPM1001 (Inaba) or pPM1002 and pPM1003 (Ogawa). Approximately 6 μg of LPS was loaded in each well. (A) The gel after silver staining; (B) a duplicate gel which was electrophoretically transferred to nitrocellulose and on which a Western blot performed with affinity-purified anti-Inaba LPS serum. The blot was developed with goat-anti-rabbit-IgG coupled with horseradish peroxidase.

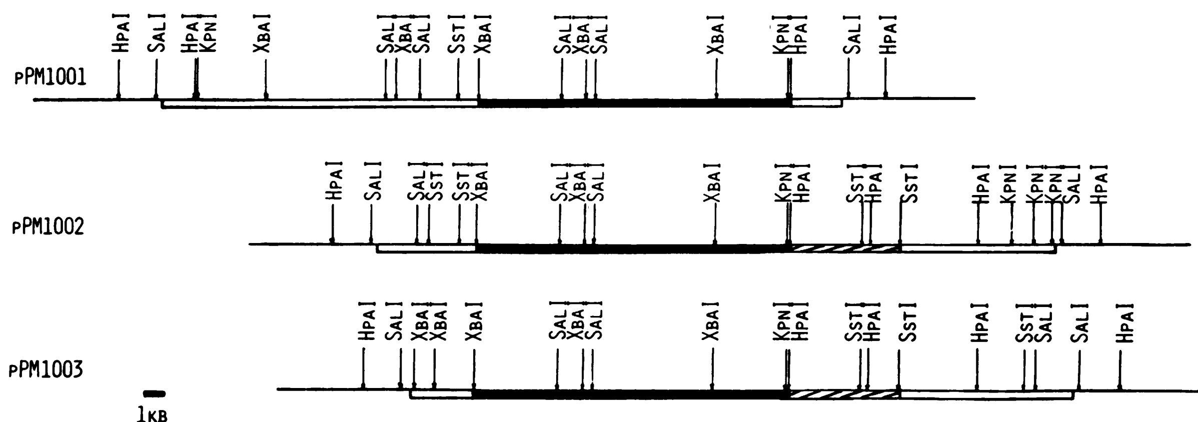

FIG. 3. Restriction analysis of the DNA contained within plasmids pPM1001 (Inaba) and pPM1002 and pPM1003 (Ogawa). The solid regions are the common DNA among all three clones; the hatched regions are DNA common to the Ogawa clones; the open regions are either nonhomologous DNA or DNA in which the extent of homology is uncertain. The thin lines correspond to the vector DNA pHC79.

E. coli K-12 DH1. Cells harboring cosmid clones were detected by plating onto nutrient media containing ampicillin. Greater than 90% of colonies were tetracycline sensitive and could be assumed to contain *V. cholerae* DNA. Random colonies showed cosmids of between 35 and 50 kb.

Restriction analysis. Restriction endonucleases were used as described by the suppliers. Analysis of the digests was by electrophoresis on either 0.8 or 1% agarose. Usually about 0.5 μg was digested for 1 h at 37°C in a total volume of 20 μl with 2 U of the appropriate endonuclease, either singly or in combination. Samples were run on gels (130 by 140 by 5 mm) submerged in TBE buffer (67 mM Tris base, 22 mM boric acid, 2 mM EDTA [pH 8.8]). DNA fragments were visualized on a transilluminating UV light source following staining with 2 μg of ethidium bromide per ml.

Hemagglutination inhibition assays. Sheep erythrocytes were sensitized with alkali-treated, purified LPS. To 100 μl of diluted antiserum (a polyclonal antiserum raised against heat-killed organisms and homologous to the LPS used to sensitize the sheep erythrocytes), containing 4 hemagglutinating units of antibody, was added 100 μl containing twofold serial dilutions of purified LPS.

Hemagglutination trays were incubated for 60 min at 37°C, and the endpoints were determined.

Protection tests. Infant mouse protection tests were performed as described previously (4). Five-day-old mice were fed 20 50% lethal doses (9.4×10^5 for strain O17 and 5.5×10^5 for strain 569B) with dilutions of antiserum. The experiment continued until the last of the controls (fed *V. cholerae* without antiserum) died.

The protective index was calculated as the reciprocal of the dilution of antiserum which gave 50% protection.

TABLE 1. Hemagglutination inhibitions

LPS source	Amt (μg) of LPS required to inhibit hemagglutination of sheep erythrocytes sensitized with LPS from:	
	569B (Inaba)	O17 (Ogawa)
V. cholerae 569B (Inaba)	0.012–0.024	0.78
V. cholerae O17 (Ogawa)	0.20	0.20
E. coli K-12 DH1(pHC79)	>100	>100
E. coli K-12 DH1(pPM1001)	0.05	3.1
E. coli K-12 DH1(pPM1003)	0.78	0.78

RESULTS

Molecular cloning of the O-antigen genes. We assumed that the synthesis of the O antigen of *V. cholerae* is determined by a gene cluster, as observed in *Salmonella* species, and that it is genetically complex. Consequently, we used cosmid cloning and in vitro packaging into bacteriophage λ as the means of reliably obtaining cloned fragments of sufficient size to enhance our chances of cloning the O-antigen gene cluster (7).

The cosmid banks were prepared (see legend to Fig. 3) and maintained frozen at −25°C on nitrocellulose filters on nutrient agar plates containing 25% glycerol (18).

Replicas of the cosmid gene banks on nitrocellulose filters were screened by colony blotting with rabbit antiserum to either live strain 569B (for the Inaba clone) or live strain O17 (for the Ogawa clones) followed by a goat-anti-rabbit-immunoglobulin G (IgG) coupled with horseradish peroxidase, as described elsewhere (11, 19, 33). About 20 strongly positive-reacting colonies from a bank of about 900 clones from 569B and 19 strongly positive-reacting colonies from 650 clones obtained from O17 were examined further.

Cell envelope material from these clones was prepared, solubilized in sodium dodecyl sulfate (SDS) and analyzed by SDS-polyacrylamide gel electrophoresis, followed by silver staining (1, 16, 20, 34). Of the clones, one derived from 569B and two derived from O17 showed a pattern typical of O-antigen material (9) (Fig. 1). These clones did not react with antisera absorbed with the homologous LPS. The plasmids in these clones were designated pPM1001 from 569B, and pPM1002 and pPM1003 from O17, respectively.

The level of expression of O-antigen material in different growth media was highly variable. Growth in brain heart infusion broth greatly suppressed the level of expression, suggesting catabolite repression, because brain heart infusion broth is rich in sugars which are not present in nutrient broth (Fig. 1). This suppression is extreme in the case of *E. coli* containing pPM1001, in which O-antigen production appeared to be eliminated. The differences among pPM1001, pPM1002, and pPM1003 may reflect different regulatory regions (see below for comparison of DNA).

LPS analysis. LPS was extracted from *E. coli* K-12 DH1 harboring pHC79, pPM1001, or pPM1003 by the hot phenol-water method (35). This material was analyzed by SDS-

TABLE 2. Infant mouse protection tests[a]

Antiserum to:	Protective index to challenge organism of:	
	569B (Inaba)	O17 (Ogawa)
V. cholerae 569B (Inaba)	700–1,200	ND[c]
V. cholerae O17 (Ogawa)	ND	680–850
E. coli K-12 DH1(pHC79)	<10[b]	<10[b]
E. coli K-12(pPM1001)	850	223

[a] Challenge tests were performed with 20 50% lethal doses simultaneously with rabbit antisera administered orally.
[b] Protective indices of <10 indicate that no protection was observed with a 1 in 10 dilution of the antiserum.
[c] ND, Not done.

polyacrylamide gel electrophoresis followed by silver staining and readily showed the production of a ladder of bands typical of O-antigen side chains on the LPS (Fig. 2). If this same material was transferred to nitrocellulose and Western blotted with antiserum to the *V. cholerae* Inaba LPS, then only this O-antigen material was recognized.

Purified LPS was also compared with LPS extracted from *V. cholerae* in hemagglutination inhibition assays to see what concentration of LPS was capable of inhibiting the agglutination of LPS-sensitized sheep erythrocytes with a constant 4 hemagglutinating units of antibody. The results (Table 1) show that the homologous serotype is better at inhibition in both cases.

The presence of plasmid pPM1001 resulted in the production of O antigen with specificity for the Inaba serotype, as judged by the amount of LPS required for inhibition, whereas pPM1003 has specificity for the Ogawa serotype. However, there was considerable cross-reaction, as was also borne out by Western blotting (Fig. 2). This was presumably due to the common A antigen because a polyclonal antiserum to the LPS was used.

The chain length of the LPS in *E. coli* K-12 was similar to that observed in *V. cholerae*, except that there was less of the higher molecular weight forms and that shorter chain length molecules were also detected (Fig. 2). The resolution of these bands into a typical ladder pattern was marked. There appeared to be a slight shift in the average length of LPS molecules with O antigen in *E. coli* K-12 (longer) compared with that in *V. cholerae* (shorter). This probably reflects a difference in the lengths of the respective core oligosaccharides. However, without more detailed knowledge of the core structure of *V. cholerae*, another possibility cannot be ruled out. It is possible that the O-antigen chains are in fact longer because of their inefficient transfer onto the *E. coli* K-12 core (17).

The results show that the enzymes involved in O-antigen biosynthesis can function in *E. coli* K-12, which itself has a defective *rfb* (O-antigen biosynthesis) region (see Mäkelä and Stocker [17] for a discussion). The ability of the LPS core of *E. coli* to be substituted with *V. cholerae* O antigen may reflect the fact that the core regions of the two organisms are related. However, the analyses demonstrate that the O antigens being produced by *E. coli* K-12 are immunologically indistinguishable from those of *V. cholerae*.

Analysis of the cloned DNA. Plasmid pPM1001 was analyzed extensively (Fig. 3), and various deletion derivatives and subclones were obtained, none of which expressed the O antigen. These results, together with those described below, imply that more than just the region of homology between the three clones is required for O-antigen expression.

Limited comparison was performed among plasmids pPM1001 and pPM1002 and pPM1003 (Fig. 3). However, there was sufficient restriction information to demonstrate that much of the DNA is identical and is flanked by regions of nonidentity. These different flanking regions probably arose during ligation because unfractionated, partially digested chromosomal DNA was used in the cloning. It can be seen that pPM1001 shares about 15 kb of DNA with pPM1002 and pPM1003 and that there is at least a further 5 kb of DNA common between the latter two Ogawa clones.

Plasmid pPM1001 possesses two large inverted repeat regions within the cloned DNA, extending outward from the central *Sal*I sites to the *Hpa*I sites near the ends of the cloned DNA (Fig. 3). This appears to be a cloning artifact and is not present as such on the chromosome (G. Morelli, M. Kamke, H. M. Ward, R. Morona, J. A. Hackett, and P. A. Manning, manuscript in preparation). It was also demonstrated that the DNA at the left end in pPM1002 and at the right end in pPM1003 represents the real contiguous chromosomal DNA.

The differences observed at the DNA level may account for variation in expression between the clones on different media. For example, genes could be expressed from a vector promoter in one case and a cloned *V. cholerae* promoter in another, and these promoters could be differentially regulated.

Interestingly, all three clones had two copies of the cloning vector joined in a head-to-head fashion. Because pHC79 replicates unidirectionally, this probably serves to repress replication and reduce plasmid copy number, a phenomenon that we observed. This seems to suggest that a high gene dosage is detrimental to the cell. In fact, plasmid pPM1004, which contains the whole of the *V. cholerae* DNA in pPM1001 cloned into pSC101 (6), a low-copy-number plasmid, is considerably more stable than pPM1001 and produces at least as much *V. cholerae* O antigen.

More detailed analyses are in progress to determine the minimum region required for O-antigen biosynthesis, the transcriptional organization of this region, and the differences betwen the Inaba and Ogawa serotypes.

Protection tests. *E. coli* K-12 strains harboring either pHC79 or pPM1001 were used to immunize rabbits to produce antisera for analysis of their protective activity in the infant mouse animal model system with either *V. cholerae* 569B (Inaba) or O17 (Ogawa) as the challenge organism (4). The results of these experiments are summarized in Table 2. They demonstrate that antisera raised against *E. coli* K-12 harboring pPM1001 compare favorably with antisera raised similarly against *V. cholerae* 569B. That is, the O antigen produced by *E. coli* K-12 harboring pPM1001 behaves antigenically like that produced by *V. cholerae* 569B and is highly protective in this model system, which is currently the best method available to us for testing protection.

DISCUSSION

We described the cloning of the *oag* regions of the *V. cholerae* chromosome of both major serotypes. Serotype variation thus occurs within a common region of DNA, as predicted from the mapping experiments performed by Bhaskaran (2; cited in reference 25). This region is large, implying genetic complexity, and the apparent lack of difference in restriction endonuclease cleavage patterns in the essential areas suggests that only minor changes are involved in serotype conversion. The Inaba and Ogawa clones will also be useful in elucidating the molecular basis for the

reciprocal serotype conversions seen in *V. cholerae* (8, 29, 30). They provide, for the first time, a basis to examine at the DNA level the changes associated with alterations in the serotype of an organism.

The results presented here also provide prospects for a new and effective cholera vaccine. It is well documented that high levels of antibodies to the O antigen are present in protective sera (5, 31) and are sufficient to provide protection in model systems. Thus, with the cloning of the determinants for the Inaba and Ogawa serotypes, it is possible to have them expressed on a suitable and safe carrier organism, which will result in production of intestinal antibodies and will enable us to determine whether such antibodies are sufficient for protection in humans. If such an organism were protective, it could be taken orally and provide immunization against cholera.

The cloned genes for O-antigen biosynthesis have also been introduced into derivatives of the oral typhoid vaccine strain *Salmonella typhi* Ty21a and an analogous *Salmonella typhimurium* strain, G30, for experiments in mice. These strains express the *V. cholerae* O antigen on their surface, and thus vaccination with them could be expected to produce antibody in the gut (13, 32). Preliminary experiments with the G30 derivatives in mice are under way. A human challenge trial with Ty21a derivatives is proposed in the near future.

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