JOURNAL OF BACTERIOLOGY, Apr. 1997, p. 2421–2425 Vol. 179, No. 7
0021-9193/97/$04.00+0

Influence of Different *rol* Gene Products on the Chain Length of *Shigella dysenteriae* Type 1 Lipopolysaccharide O Antigen Expressed by *Shigella flexneri* Carrier Strains

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Received 28 October 1996/Accepted 23 January 1997

Introduction of the *rol* genes of *Shigella dysenteriae* 1 and *Escherichia coli* K-12 into *Shigella flexneri* carrier strains expressing the heterologous *S. dysenteriae* type 1 lipopolysaccharide resulted in the formation of longer chains of *S. dysenteriae* 1 O antigen. In bacteria producing both homologous and heterologous O antigen, this resulted in a reduction of the masking of heterologous O antigen by homologous lipopolysaccharide and an increased immune response induced by intraperitoneal immunization of mice by recombinant bacteria. The *rol* genes of *S. dysenteriae* 1 and *E. coli* K-12 were sequenced, and their gene products were compared with the *S. flexneri* Rol protein. The primary sequence of *S. flexneri* Rol differs from both *E. coli* K-12 and *S. dysenteriae* 1 Rol proteins only at positions 267 and 270, which suggests that this region may be responsible for the difference in biological activities.

Shigella dysenteriae 1 is a causative agent of bacillary dysentery, a major public health problem in developing countries (6). The serotype-specific lipopolysaccharide (LPS) appears to be the main antigen responsible for a protective immune response. The LPS is a major component of gram-negative bacterial outer membrane and consists of three structural regions: (i) the hydrophobic lipid A, which anchors LPS to the outer membrane; (ii) the core oligosaccharide; and (iii) the O-antigen chain, consisting of repeating oligosaccharide units that are serotype specific (16). One strategy to develop effective vaccines consists of expression of the O antigen in a carrier strain. A prerequisite to achieve an efficient immune response is the accessibility of the heterologous LPS to the immune system. However, in a bivalent vaccine strain, heterologous O antigen may be masked by longer chains of the homologous O antigen (1, 11, 35), and it is known that long LPS chains sterically hinder shorter chains from binding macromolecules (17).

Monomeric units of the O antigen are assembled on the antigen carrier on the cytoplasmic face of the inner membrane. After translocation to the periplasmic face of the membrane, the monomers are polymerized by the O-antigen polymerase (*rfc* gene product) and polymers (or monomers) are subsequently transferred to lipid A core molecules by the *rfaL*-encoded O-antigen core ligase (26). The biological activities of the O antigen have been correlated with its length and distribution on the bacterial outer membrane (13, 17). The mean length of O-antigen chains is specific for each serotype and exhibits a nonrandom distribution with a preference for longer chains (3). This pattern cannot be explained by fixed probabilities for extension by polymerase or ligation to lipid A core, which would lead to a continuous ladder with a decreasing amount of LPS with increasing chain length (12).

A gene (*rol* [regulator of O length] or *cld* [chain length determinant]) whose product regulates O-antigen chain length has been described elsewhere (2–5, 25). The length distribution of LPS molecules as determined by the 36-kDa Rol protein exhibits a modal (i.e., preferred length) or bimodal pattern. A Rol protein of one serotype may function in different serotypes and thereby alter the typical chain length distribution. The *rol* gene is located adjacent to the *rfb* cluster, which encodes proteins required in O-antigen synthesis, and has been characterized for *Escherichia coli* O75, *E. coli* O111, *Salmonella* spp., and *Shigella flexneri* serotype Y (2–5, 7, 25, 29). Similar genes were also found adjacent to the *rfb* region of *Yersinia pseudotuberculosis*, in the *rfe* locus of *E. coli* K-12, and on the natural 3.05-kb plasmid pHS-2 of *S. flexneri* serotype 2a (3, 19, 31).

The chromosomal *rfb* and plasmid-borne *rfp* genes responsible for biosynthesis of the *S. dysenteriae* type 1 O antigen have been cloned and combined as an *rfp-rfb* gene cassette (32). When plasmids containing this cassette were introduced into *E. coli* K-12 (32), *Salmonella enterica* serovar typhimurium (27), or *S. flexneri* Y (10), the recombinant strains efficiently expressed the heterologous O antigen with a modal length distribution that was controlled by the host carrier strain *rol* gene. On the other hand, the O-antigen chain length was not regulated when the cassette was expressed in *E. coli* SΦ874 (23), which lacks a segment of the *rfb* locus which includes a determinant of O-antigen chain length regulation (2, 5, 7, 25), indicating that the *S. dysenteriae* 1 *rol* gene is not present on the *rfp-rfb* cassette.

To develop vaccine candidates against *S. dysenteriae* 1, the *rfp-rfb* gene cassette was introduced into the attenuated *S. flexneri* vaccine strain SFL124 (18, 34) and its rough (O antigen lacking) derivative SFL124-27 (10, 20), resulting in the expression of both homologous and heterologous O antigens in SFL124 and only heterologous O antigen in SFL124-27 (10, 21). The developed bivalent *S. flexneri* Y-*S. dysenteriae* 1 strains synthesized shorter-than-normal chains of the heterologous O antigen (see below). We investigated the usefulness of providing different *rol* genes to increase the chain length of the

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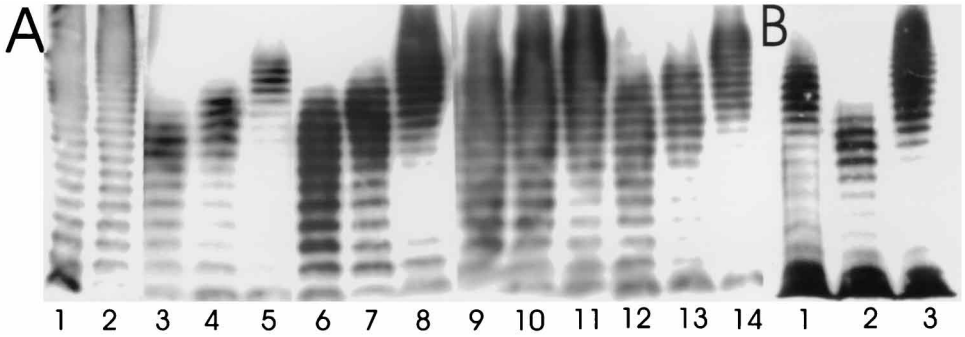

FIG. 1. Synthesis of *S. dysenteriae* 1 O antigen by recombinant *S. flexneri* carrier strains and wild-type *S. dysenteriae* 1 carrying heterologous *rol* genes. LPS of recombinant *S. flexneri* SFL124 and SFL124-27 (A) and *S. dysenteriae* 1 (B) strains was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and developed by immunoblotting with polyclonal antibodies against *S. dysenteriae* 1 LPS (A) or by silver staining (B) (33). (A) Lane 1, wild-type *S. dysenteriae* 1 strain W30864; lane 2, *E. coli* DH5α (pSDM4); lane 3, SFL124::Tn(*rfp-rfb*)-6; lane 4, SFL124::Tn(*rfp-rfb*)-6 (pSK6); lane 5, SFL124::Tn(*rfp-rfb*)-6 (pSK7); lane 6, SFL124::Tn(*rfp-rfb*)-7; lane 7, SFL124::Tn(*rfp-rfb*)-7 (pSK6); lane 8, SFL124::Tn(*rfp-rfb*)-7 (pSK7); lane 9, SFL124-27::Tn(*rfp-rfb*)-8; lane 10, SFL124-27::Tn(*rfp-rfb*)-8 (pSK6); lane 11, SFL124-27::Tn(*rfp-rfb*)-8 (pSK7); lane 12, SFL124-27::Tn(*rfp-rfb*)-39; lane 13, SFL124-27::Tn(*rfp-rfb*)-39 (pSK6); lane 14, SFL124-27::Tn(*rfp-rfb*)-39 (pSK7). (B) Lane 1, *S. dysenteriae* 1 strain W30864; lane 2, W30864 (pSK8); lane 3, W30864 (pSK7). pSK6 and pSK7 are recombinant plasmids containing the *rol* genes of *S. dysenteriae* 1 and *E. coli* K-12, respectively.

heterologous O antigen expressed by the recombinant *S. flexneri*::Tn(*rfp-rfb*) strains.

Chain length distribution of *S. dysenteriae* 1 O antigen in recombinant *S. flexneri* carrier strains and in *S. dysenteriae* 1. LPS was isolated as previously described (15) from *S. dysenteriae* 1 strain W30864 (wild type), *E. coli* K-12 DH5α harboring plasmid pSDM4 (*rfp-rfb* cassette [9]), and recombinant *S. flexneri* strains and subjected to electrophoresis on 15% polyacrylamide gels (polyacrylamide gel electrophoresis [24]). After Western blotting, the LPS was detected with a rabbit polyclonal antiserum against *S. dysenteriae* 1 LPS (Behringwerke, Marburg, Germany) as described previously (10). *S. dysenteriae* 1 LPS isolated from W30864 and *E. coli* DH5α (pSDM4) exhibited long O-antigen chain length (≥20 units), indicating that the *rol* genes of both strains mediate the preferred transfer of longer chains to the lipid A core (Fig. 1A, lanes 1 and 2). LPS from *S. flexneri* SFL124 or SFL124-27 carrying the *rfp-rfb* cassette integrated in either the chromosome or the invasion plasmid exhibited, however, much shorter chain lengths (11 to 15 units [Fig. 1A, lanes 3, 6, 9, and 12]). This suggests that the *rol* gene of *S. flexneri* serotype Y determines a length distribution characterized by shorter O chains, as has also been observed for the homologous LPS, where most of the O-antigen molecules consist of 10 to 17 repeat units (29).

Since the *rfp* genes are encoded on a multicopy plasmid in *S. dysenteriae* 1 but are present in only one copy in the recombinant *S. flexneri* strains, the differences in chain length might in part be due to a reduced synthesis of heterologous O antigen by the carrier strain. Slightly longer O-antigen chains were in fact synthesized by *S. flexneri* SFL124 bacteria carrying the multicopy pSDM4 plasmid, in which homologous and heterologous O antigens were expressed in a 1:1 ratio, although competition between both O-antigen types for ligation to the lipid A core occurred (10). However, no differences in *S. dysenteriae* 1 O-antigen chain length were observed between SFL124-27 (pSDM4) and SFL124-27::Tn(*rfp-rfb*)-8 (integration into virulence plasmid [data not shown]), ruling out a major role for *rfp* copy number in the determination of chain length. In addition, the *S. dysenteriae* 1 O-antigen chain length in SFL124-27 (pSDM4) was not as high as in *S. dysenteriae* 1 or in *E. coli* DH5α (pSDM4) (data not shown), suggesting that the observed differences in chain length distribution are due to the nature of the *rol* gene product.

Cloning of the *rol* genes from *E. coli* K-12, *S. dysenteriae* 1, and *S. flexneri*. The *rol* genes of different organisms exhibit a high degree of homology (29). We therefore designed two primers based on the *rol* gene sequence of *E. coli* O75 (4) to amplify *rol* genes by PCR. The forward primer (5′-TTTTTAT CACTTATCCTATAGCA-3′) was complementary to the −35 promoter region, whereas the reverse primer (5′-ACGGCAT CAGTGAGTTGCGTG-3′) encompassed the stop codon (underlined) of the adjacent *his* region, which is oriented in the opposite direction from that of *rol* (4, 8). Single colonies of *E. coli* DH5α (38), *S. dysenteriae* W30864 (36), and *S. flexneri* SFL124 (18) (Table 1) were suspended in 200 μl of H_2O and heated at 95°C for 12 min, and 10 μl of each suspension was used as template for PCR amplification with the Perkin-Elmer GeneAmp Kit. The PCR products were ligated to the pCR II vector of the TA cloning system (Invitrogen, Leek, The Netherlands) and transformed into *E. coli* INVαF′ (Invitrogen). The resulting recombinant plasmids pSK6 (*S. dysenteriae* 1 *rol* gene), pSK7 (*E. coli* K-12 *rol* gene), and pSK8 (*S. flexneri* Y *rol* gene) were used for subsequent studies.

The inserts containing the *rol* gene of pSK6 (1,170 bp) and pSK7 (1,171 bp) were completely sequenced in both directions with the Taq DyeDeoxy Terminator Cycle Sequencing Kit from Applied Biosystems (Foster City, Calif.). DNA sequence data were obtained from the ABI Model 373A Automated Sequencer. The sequence of the *rol* gene of SFL124, which is the *aroD* derivative of SFL1 (18), showed no difference from the published sequence of this latter strain (29). The DNA sequences of the *S. dysenteriae* 1, *S. flexneri* Y, *E. coli* K-12, and *E. coli* O75 *rol* genes were highly homologous starting upstream from the −35 region and ending in the region of the transcriptional terminator. Interestingly, the *rol* sequences of *S. dysenteriae* 1 and *E. coli* K-12 were approximately 90 bp shorter in the 3′ noncoding region than the sequences of *S. flexneri* and *E. coli* O75. A similar result has been observed for the *rol* sequence of *S. enterica* LT2 (4) and *E. coli* O111 (3).

The amino acid sequences translated from the *rol* genes of *S. dysenteriae* 1, *S. flexneri*, and *E. coli* K-12 were compared (Fig. 2). The Rol protein of *E. coli* K-12 (326 residues, additional glycine at position 221) is 1 amino acid longer than the other proteins (Fig. 2). All three proteins were almost identical, with identity levels ranging from 97 to 98%. Differences in the amino acid sequences occurred at only nine positions, although

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant genotype or characteristics[a]	Reference(s) or source
Strains		
S. flexneri SFL124	Δ*aroD*, serotype Y, Sf^+	18
S. flexneri SFL124-27	Δ*aroD*, rough mutant of SFL124, Sf^-	10
SFL124::Tn(*rfp-rfb*)-6	Δ*aroD*, Tn(*rfp-rfb*) on chromosome, Sf^+, Sd^+	21, 34
SFL124::Tn(*rfp-rfb*)-7	Δ*aroD*, Tn(*rfp-rfb*) on virulence plasmid, Sf^+, Sd^+	21
SFL124-27::Tn(*rfp-rfb*)-8	Δ*aroD*, Tn(*rfp-rfb*) on virulence plasmid, Sf^-, Sd^+	20
SFL124-27::Tn(*rfp-rfb*)-39	Δ*aroD*, Tn(*rfp-rfb*) on chromosome, Sf^-, Sd^+	20
S. dysenteriae W30864	Sm^r, serotype 1	36
E. coli K-12 DH5α	*endA1 recA1 hsdR17*(r_K^- m_K^+) *supE44 thi-1 gyrA96* Φ80d *lacZ*ΔM15 Δ(*lacZYA-argF*)*U169* d^-	38
E. coli K-12 INVαF′	F′ *endA1 recA1 hsdR17*(r_K^- m_K^+) *supE44 thi-1 gyrA96 relA1* Φ80 *lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 deoR* λ^-	Invitrogen
Plasmids		
pSDM4	13.9-kb *rfp-rfb* cassette in pUC19	9
pCR II	Cloning vector	Invitrogen
pSK6	1.17-kb PCR product with *S. dysenteriae* 1 *rol* in pCR II	This study
pSK7	1.17-kb PCR product with *E. coli* K-12 DH5α *rol* in pCR II	This study
pSK8	1.26-kb PCR product with *S. flexneri* SFL124 *rol* in pCR II	This study

[a] Sf, production of *S. flexneri* O antigen; Sd, production of *S. dysenteriae* 1 O antigen; +, positive; −, negative.

five involved conservative substitutions (positions 62, 217, 224, 254, and 270 [Fig. 2]). Of the other four positions, two involved nonconservative changes (positions 267 and 285) located in a predicted α-helix (positions 252 to 283) close to the COOH-terminal hydrophobic domain.

The mode of action of the Rol/Cld proteins is unclear at present. One model (3) suggests that the Cld protein forms a complex with the O-antigen polymerase (Rfc protein) and acts as a molecular clock, switching this enzyme between an extension-facilitating and a transfer-facilitating state. In contrast, Morona et al. (29) suggested that the Rol protein is a molecular chaperone which interacts with the RfaL protein (O antigen-core ligase) to assemble a complex. This complex would give a specific RfaL/Rfc ratio, thereby modulating the ligation kinetics and resulting in a nonrandom O-antigen chain length distribution. In either case, Rol proteins would need to interact with proteins from different strains which show little sequence

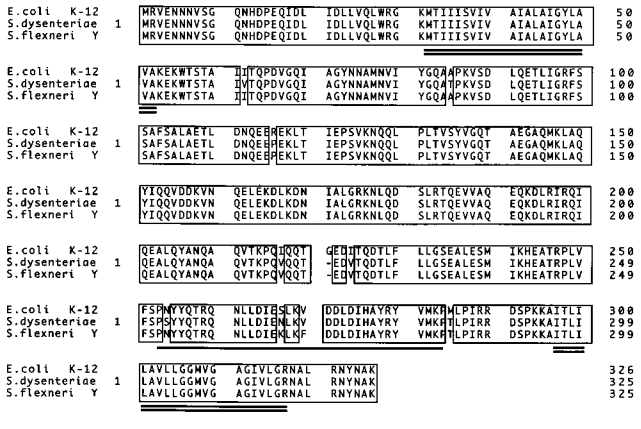

FIG. 2. Alignment of the primary Rol protein sequences of *E. coli* K-12 DH5α, *S. dysenteriae* 1 strain W30864, and *S. flexneri* SFL1 (29). The alignment was performed with the Gene Works 2.45 program (IntelliGenetics, Inc.). Regions with identity are boxed, and the predicted α-helix (positions 252 to 283, underlined) and the potential membrane-spanning hydrophobic regions (double underlined) are indicated.

similarity. The Rfc (28) and the RfaL (22) proteins do, however, exhibit similar hydropathy plots, suggesting structural similarities. Two strongly conserved motifs occur near the amino-terminal transmembrane segment of different Rol proteins and could be potential points of interaction with either Rfc or RfaL (31), which is an integral inner membrane protein. The differences in amino acid sequences of the *E. coli* K-12, *S. dysenteriae* 1, and *S. flexneri* Rol proteins were localized neither within these conserved motifs nor within hydrophobic, putative membrane-spanning domains at positions 32 to 52 and 296 to 316 (29, 31). The primary sequence of *S. flexneri* Rol differs from both *E. coli* K-12 and *S. dysenteriae* 1 Rol proteins only at positions 267 and 270. This suggests that such exchanges within the predicted α-helix may be responsible for the observed differences in the biological activity (see below).

Influence of the different *rol* genes on the chain length of *S. dysenteriae* 1 O antigen. Plasmids pSK6 and pSK7 containing the *rol* genes of *S. dysenteriae* 1 and *E. coli* K-12 were introduced into the recombinant *S. flexneri* strains (Table 1) by electroporation (30). Both Rol proteins directed the synthesis of longer chains of *S. dysenteriae* 1 O antigen (Fig. 1A), with the *E. coli* protein causing the longest to be produced (from 17 to ≥20 units).

The *rol* genes of *S. flexneri* and *E. coli* K-12 were introduced into the wild-type strain *S. dysenteriae* 1, and their effect on chain length distribution was examined. *S. dysenteriae* 1 synthesized shorter chains when transformed with the *rol* gene of *S. flexneri* (11 units) and longer chains (≥20 units) when transformed with the *rol* gene of *E. coli* K-12 (Fig. 1B). This confirmed that the *S. flexneri rol* gene determines shorter O-antigen chains, whereas the *S. dysenteriae* 1 and particularly the *E. coli* K-12 *rol* genes direct the polymerization of longer chains.

The three analyzed *rol* genes exhibit a high degree of homology. Therefore, to assess whether the observed differences in the O-antigen chain length were in part due to the higher *rol* copy number, we introduced the plasmid-encoded *S. flexneri rol* gene into SFL124, SFL124::Tn(*rfp-rfb*), and SFL124-27::Tn(*rfp-rfb*). The lengths of both the homologous and the heterologous O antigens synthesized by the carrier strains were not detectably

influenced (data not shown). This ruled out that the effect was due to the presence of the *rol* gene in multicopy, confirming that the major role is played by the source of the gene.

In the tested recombinant strains, the homologous and heterologous O-antigen units are polymerized by the Rfc protein and then linked to the lipid A core by the *S. flexneri* RfaL protein. It is difficult to imagine how the almost-identical Rol proteins of *S. flexneri* Y, *S. dysenteriae* 1, and *E. coli* K-12 are able to direct the observed differences in *S. dysenteriae* 1 O-antigen chain length distribution by interacting with Rfc and/or RfaL proteins. In a yet-unknown manner, minimal differences in the Rol protein sequence must have striking effects. The amino acid exchanges in the three Rol proteins compared in this study are occurring mainly in the putative periplasmic domain of the protein, suggesting that this domain is very important for its regulatory activity.

The expression of longer heterologous LPS chains should reduce masking by homologous O antigen in bivalent vaccine candidates. A simultaneous elongation of the homologous LPS chains would, however, tend to negate any such effect. The influence of the *rol* genes of *S. dysenteriae* 1 and *E. coli* K-12 in SFL124 and SFL124::Tn(*rfp-rfb*) on the chain length of homologous O antigen was therefore examined and found to provoke the synthesis of longer chains though the proportional increase in chain length of homologous antigen was less than that for the heterologous antigen. The result was an overall reduction in the chain length difference between homologous and heterologous O antigens (data not shown). To assess whether the reduced difference in chain length resulted in a reduced masking of the heterologous O antigen, we performed microscopic analysis of intact bacteria by immunofluorescence. The results showed that those recombinant clones harboring the plasmid containing the *E. coli rol* gene were characterized by a brighter fluorescence signal (not shown).

Immunogenicity of recombinant *S. flexneri* strains containing the plasmid-encoded *E. coli* K-12 *rol* gene. We determined whether the presence of longer *S. dysenteriae* 1 O chains resulting from introduction of the *E. coli* K-12 *rol* gene into the recombinant *S. flexneri* strains affected the immunogenicity of such strains. Mice (groups of five animals) were immunized by three intraperitoneal injections of 0.2×10^8 to 1.0×10^8 heat-killed bacteria (60°C, 1 h) of the recombinant strains SFL124::Tn(*rfp-rfb*)-7 and SFL124::Tn(*rfp-rfb*)-7 (pSK7) and the wild-type *S. dysenteriae* 1 strain on days 0, 14, and 28. A group of nonimmunized mice was included as control. Two weeks after the last immunization, the mice were sacrificed, blood samples were collected, and titers of antibody against LPS of *S. dysenteriae* 1 were determined by enzyme-linked immunosorbent asay. Microtiter plates were coated with LPS (1 μg per well) prepared by the method of Westphal and Jann (37) and used to assay antibodies of serially diluted mouse sera as described previously (14). An enhanced antibody response was seen in the animals immunized with the bivalent strain SFL124::Tn(*rfp-rfb*) containing the *E. coli* K-12 *rol* gene, presumably due to a reduced masking of the heterologous LPS (Fig. 3).

The presentation of heterologous O antigens on the surface of a well-characterized live vaccine strain is a promising strategy in vaccine development. Our data suggests that the efficacy of such vaccine candidates can be optimized by providing heterologous *rol* genes to increase O-antigen chain length and reduce masking by homologous O antigen.

Nucleotide sequence accession numbers. The sequences of the *rol* genes of *S. dysenteriae* 1 and *E. coli* K-12 appear in the EMBL data library under the accession numbers Y07560 and Y07559.

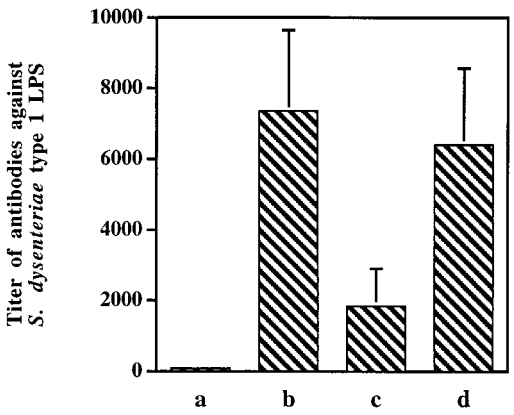

FIG. 3. Effect of *E. coli* K-12 *rol* gene on the immunogenicity of the carrier strains. Titers of serum antibodies against *S. dysenteriae* 1 LPS elicited by immunization of mice with wild-type *S. dysenteriae* 1 strain W30864 (b), *S. flexneri* SFL124::Tn(*rfp-rfb*)-7 (c), and SFL124::Tn(*rfp-rfb*)-7 (pSK7) (d) bacteria were determined by enzyme-linked immunosorbent assay. A nonimmunized group (a) was included as control. The antibody titers are expressed as the geometric means of the maximal reciprocal dilutions which resulted in an optical density at 490/595 nm equal to or greater than the cutoff value of 0.1 after 1 h of incubation with the substrate; standard errors are represented by vertical lines.

We thank A. A. Lindberg for providing *S. flexneri* SFL124 and SFL124-27, H. Watanabe for providing *S. dysenteriae* 1 strain W30864, and B. Karge and A. Krüger for technical assistance.

K.N.T. gratefully acknowledges generous support from the Fonds der Chemischen Industrie.

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