Simultaneous expression of smooth and rough phase properties related to lipopolysaccharide in a strain of *Brucella melitensis*

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Summary. *Brucella* strains exhibit either a rough (R) or a smooth (S) colonial phase identifiable by bacteriological methods. This depends on the biosynthesis and translocation to the surface in S but not in R strains, of the O-polysaccharide chain of the lipopolysaccharide (LPS) molecule. *B. melitensis* biovar 1 strain EP exhibited simultaneously both S and R characteristics in relation to colonial morphology, agglutination by monospecific anti-M and anti-R sera, activity of bacteriophages lytic for rough *Brucella* spp. (phage R/C) and for smooth *B. melitensis* (phage Iz). *B. melitensis* strain EP expressed fewer O-chains with a similar distribution of molecular weights than *B. melitensis* reference strain 16M by SDS-PAGE and immunoblotting, but higher amounts of R-LPS. Quantitative determination of S-LPS by a turbidimetric latex inhibition immunoassay with monoclonal antibodies confirmed the limited expression of S-LPS in strain EP. As with other gram-negative bacteria, the phenomenon could be attributed to a deficiency in one step of the biosynthetic assembly of the O-chains.

Introduction

The genus *Brucella* includes species described as “smooth” (S) or “rough” (R) depending on the colonial morphology and related properties. Some of the methods used currently to identify *Brucella* isolates and to characterise and control reference strains rely upon the characteristics of the bacterial cell surface. Thus, direct observation of colonies, crystal violet staining, agglutination in the presence of acriflavine, serotyping and phage sensitivity are used to differentiate the S from the R phase.^{1a}

In Enterobacteriaceae, phase variation is related to mutation of steps in the biosynthesis of lipopolysaccharide (LPS) molecules and their translocation to the surface of the cell.² In the S colonial phase, brucella cells express the complete LPS (S-LPS) with the O-chain, plus LPS molecules without the O-chain (R-LPS)³ but the R phase cells express only the R-LPS. Phase variation in brucellae^{1b} is also related to the two major (A and M) antigenic specificities used in the classification of *Brucella* spp.⁴ These specificities reside in the O-chain of S-LPS.⁵ Although the use of the above techniques allows the correct identification of the majority of *Brucella* isolates as S or R phase, some

strains do not give clear-cut results, and are, therefore, difficult to classify. Descriptions of such strains of *Brucella*⁶ and bacteria of other genera⁷ have been reported but the methods used to investigate them have been limited.

The characterisation of a strain of *B. melitensis* biovar 1 of ambiguous colonial phase, strain EP, by traditional bacteriological and novel methods, together with molecular analysis of LPS by SDS-PAGE and quantitative immunoturbidimetric determination of S-LPS, showed clearly that this strain displayed an unusual level of expression of both S-LPS and R-LPS. The relative merits of the procedures available for determination of colonial phase in brucellae in the context of our observations on *B. melitensis* strain EP are discussed in this paper.

Materials and methods

Bacterial strains

B. melitensis strain EP, isolated from a patient with acute brucellosis, was obtained from Dr E. J. Young⁸ and is conserved in the *Brucella* culture collection, INRA-Nouzilly, France as No. BCCN 87.91. The other *Brucella* strains used in this study were obtained from the same collection: *B. abortus* S99, a smooth strain used for the elaboration of brucella antigen for

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diagnosis;^{1c} *B. melitensis* B115, a rough strain used for preparation of brucellin;^{1d} *B. melitensis* 16M, the type strain of *B. melitensis* and reference of the biovar 1 of this species;^{1e} *B. melitensis* Rev-1(R), a dissociated clone obtained from the smooth, vaccinal strain Rev 1; and *B. abortus* 544, a smooth virulent strain, currently used for challenge in protection experiments.

Bacteriological methods

The strains were checked for purity, species and biovar characterisation by standard procedures^{1a} if not otherwise indicated.

Colonial morphology of strain EP was assessed in cultures grown both on Trypticase Soy Agar (bio-Mérieux, Marcy l'Etoile, France) supplemented with yeast extract (Difco) 0.1% (TSAYE medium) and on Blood Agar Base No. 2 (Oxoid; BAB medium). Methods for the observation of colonial morphology were those described by Alton *et al.*^{1b}, i.e., direct observation of colonies by obliquely reflected light, acriflavine test and staining of colonies by crystal violet.

Izarnagar (Iz) phage,⁹ lytic for smooth *B. melitensis* strains and R/C phage,¹⁰ lytic for rough brucellae were used to confirm the dual smooth-rough properties of strain EP. The Iz clone used in this laboratory was derived from a sample of Iz₁ phage provided by M. J. Corbel in 1985. It was propagated on S phase *B. melitensis* strain 16M and has subsequently proved lytic for S phase cultures only. As most smooth strains of *B. melitensis* were found to be lysed by this phage, it was used in this study as a marker for the smooth character.

Serotyping by slide agglutination was performed with monospecific A and M polyclonal antisera prepared according to the method of Jones,¹¹ and with an anti-R specific polyclonal serum prepared according to the method of Corbel *et al.*¹² Agglutination patterns were interpreted as reported elsewhere.¹³

FACS analysis

B. melitensis strain EP was grown as indicated for PK-LPS extracts. Once harvested and inactivated, *c.* 0.5×10^9 cells were distributed in Eppendorf vials in which they were washed twice with phosphate buffered saline (PBS). Bacteria were then resuspended in 200 μ l of appropriate dilutions of monoclonal antibodies (MAbs) and incubated at 37°C for 1 h. After three washings, cells were incubated with a 1 in 40 dilution of FITC-conjugated pig anti-mouse immunoglobulins (Nordic), at 37°C for 1 h. Finally, the cells were washed three times, resuspended in 1 ml of PBS and analysed. Cells incubated with the FITC conjugate alone served as controls.

The fluorescence emissions of 50 000 FITC-stained brucella cells were quantified with a FACScan flow cytometer (Becton Dickinson Immunocytometry

Systems, Mountain View, CA, USA). The gates of the detectors were set to exclude large bacterial aggregates from the analysis.

Proteinase K-LPS (PK-LPS) extracts

To study LPS electrophoretic patterns and immuno-reactivity, digested, whole-cell brucella lysates were prepared as follows. Cells from cultures grown on TSAYE or BAB slants at 37°C for 24 h were suspended in PBS containing Tween 80 0.05 w/v (PBS-T), pH 6.85, and washed twice in this medium followed by resuspension at 3×10^{10} cfu/ml in lysis solution (SDS 1%, glycerol 2% v/v, 0.0625 M Tris-HCl, pH 6.8). After boiling for 10 min the temperature was adjusted to 50°C and 0.625 mg of proteinase K (Boehringer Mannheim GmbH, Germany) was added to 1 ml of lysate. The sample was digested at 50°C for 2 h and then at 20°C for 18 h. For some assays, cell number and enzyme concentration were increased four-to-six times. PK-LPS extracts were kept frozen at -20°C until required. For LPS quantitative determination, PK-LPS extracts were prepared from lyophilised cells as reported elsewhere.¹⁴

R-LPS and S-LPS extracts

Cells from *B. melitensis* strain B115 were cultured in the same medium and the conditions specified by USDA, Ames, Iowa as described previously.¹¹ Washed cells suspended in 0.15 M NaCl were dehydrated in acetone as for brucellin production.^{1d} R-LPS was extracted from cells by the phenol-chloroform-petroleum ether-water method of Galanos *et al.*¹⁵

S-LPS of *B. abortus* S99 was prepared by the phenol-water method of Leong *et al.*¹⁶

SDS-PAGE

PK-LPS samples were analysed by SDS-PAGE with the discontinuous buffer system of Lugtenberg¹⁷ with some modifications.¹⁸ Homogeneous separating gels (140 × 140 × 0.75 mm) of acrylamide 11% were cast and overlaid by an acrylamide 4% stacking gel. Alternatively, acrylamide 11–35% linear gradient gels (140 × 140 × 1.5 mm) were prepared to analyse R-LPS. Ten μ l of each extract were carefully loaded and run for 120 min at 40 mA/gel for homogeneous gels, and for 300 min at 30 mA/gel for the gradient gels. The gels were stained for carbohydrates by the periodic acid-silver nitrate method of Dubray and Bézard.¹⁸

Monoclonal antibodies (MAbs)

To screen for epitopes present on the O-chain or in the core of the LPS, the A61/16C10/G12, B66/04F9 and A68/12/F12/G12 and anti-brucella S-LPS MAbs, specific for the M, A and common epitopes of S-LPS, respectively,¹⁹ and the A68/03F03/D05 MAb specific for R-LPS²⁰ were used. They were screened for

Table. Main differential characteristics of *B. melitensis* strain EP and strain 16M

Test	Result with <i>B. melitensis</i> strain	
	16M	EP
Observation of colonies		
direct	S	S
crystal violet strain*	-	+
Agglutination		
acriflavine	-	+
monospecific serum		
anti-A	-	-
anti-M	+	+
anti-R	-	+
Lysis by phages		
Iz	+	+
R/C	-	+
LPS expression level†		
S-LPS	10	1
R-LPS	+	++

S, indicates colonies with a smooth, glossy surface.

* +, uptake; -, no uptake, of crystal violet by the colonies.

†For S-LPS, figures express relative amounts as determined by the immunoturbidimetric assay. For R-LPS, + and ++ denote relative abundance as visualised in SDS-PAGE after silver staining.

Fig. 1. FACS analysis of strain EP. Fluorescence histograms show the distribution of the binding of (a) control serum (FITC-conjugated swine anti-mouse serum), (b) anti-R-LPS MAb A68/03F03/D05 and (c) anti-OPS MAb A68/12/F12/G12. Fluorescence intensity was plotted in relative units in a logarithmic scale.

specificity by the automated particle counting immunoassay (PACIA) and by an enzyme-linked immunosorbent assay (ELISA).²¹

Immunoblotting

After electrophoresis, gel slabs were soaked for 15 min in blotting buffer (39 mM glycine, 48 mM Tris, SDS 0.0375%, methanol 20% v/v) then transferred to 0.2- μ m pore size nitrocellulose membranes (BA83 Schleicher and Schuell, Dassel, Germany) in an LKB 2117 Multiphor II electrophoresis apparatus operated for 70 min at 0.8 mA/cm². Blocking was by overnight incubation in skimmed milk 1% in 20 mM Tris, 500 mM NaCl (TBS) at room temperature with continuous gentle agitation.

Blots were incubated for 90 min with (i) rabbit anti-M monospecific serum, (ii) rabbit anti-A monospecific serum, (iii) rabbit anti-R monospecific serum, (iv) anti-M MAb, (v) anti A-MAb or (vi) anti-R MAb at previously determined dilutions in skimmed milk 0.3%-TBS.

The secondary antibody was either an anti-rabbit or anti-mouse biotinylated sheep F(ab')₂, (Amersham International) and incubation was at room temperature for 90 min. Bound antibody was detected by incubating with streptavidin-peroxidase (Amersham International) at room temperature for 60 min and the peroxidase activity was revealed by the HRP Color Development Reagent (BioRad).

In some experiments, gels were stained for carbohydrates after blotting, as indicated elsewhere in this section.

LPS-coated latex

The details of the technique for coating were described in detail previously.²¹ Briefly, 50 μ l of K109 latex suspension (Rhône-Poulenc, Courbevoie, France) 10% w/v were incubated with 100 μ g of standard brucella S-LPS (f5) in 400 μ l of 0.1 M glycine, 0.17 M NaCl, pH 9.2 (GBS) diluted 1 in 5, sonicated for 1 min at 30 W, stabilised by adding 200 μ g of bovine serum albumin (BSA) in GBS 1 in 5 and incubated at room temperature for 20 min. The pellet obtained after centrifugation at $1.2 \times 10^4 g$ for 10 min was suspended in 1 ml of SDS 1%, resuspended and incubated for 1 h at 37°C. It was then washed twice with 1 ml of GBS 1 in 5, once with BSA 1%, 50 mM EDTA in GBS and finally suspended in 1 ml of this buffer. This 0.5% stock latex suspension was stored at -20°C until use. To enhance agglutination, 4 μ l of rheumatoid factor serum were added to 1 ml of latex 0.05%.

LPS quantitative determination

To compare the relative amounts of LPS of the EP strain and the reference *B. melitensis* strain 16M, a turbidimetric latex inhibition immunoassay was performed. PK-LPS extracts were prepared from an equal amount (dry weight of lyophilised cells) as indicated elsewhere.¹⁴ The samples were first diluted 1 in 2 in BSA 20%, 0.1 M glycine, 0.17 M NaCl, Tween 80

Fig. 2. SDS-PAGE of whole-cell lysates from smooth *B. abortus* 544 (lane 1), smooth *B. melitensis* 16M (2), *B. melitensis* EP (3 and 4) and *B. melitensis* Rev-1 rough variant (5–7). Concentrations of samples were the same except in lanes 4 and 6 in which the amount loaded was increased six-fold, and in lane 7 in which it was reduced five-fold. Lanes 5 and 6 contain purified R-LPS from *B. melitensis* B115. Molecular mass markers, in lane 8, were: bovine serum albumin, 66 kDa; ovoalbumin, 45 kDa; glyceraldehyde 3-phosphate dehydrogenase, 36 kDa; carbonic anhydrase, 29 kDa; trypsinogen, 24 kDa; soybean trypsin inhibitor, 20.5 kDa.

0.1%, 0.05 M EDTA, pH 9.2, (BSA 10%-GBS), serially 1.5-fold diluted in filtered BSA 1%-GBS and distributed in flat-bottomed microtitration plates (Greiner PS-Mikrotiterplate, 96K, F-form) at 40 μ l/well. Then, 40 μ l of a previously titrated dilution of MAb were added and the plates were shaken continuously for 30 min at room temperature. The latex suspension (40 μ l/well) was distributed and the plates were shaken for a few seconds. The differences (Ao – At) between the absorbances (405 nm) recorded immediately after latex addition (Ao) and after continuous agitation for 15 min (At) were calculated automatically (Titertek Multiskan MCC 340/MKII, Flow). The data were plotted against the PK-LPS dilutions, by transforming mean Ao–At of triplicates in inhibition percentages. The decrease of the absorbance of the latex suspension provoked by the agglutination of the beads, is inversely proportional to the S-LPS concentration in the PK-LPS samples.

Results

Bacteriological identification

Strain EP was identified as *B. melitensis* biovar 1 on the basis of the results of the tests recommended for the identification and typing of *Brucella* strains. Standard procedures—lysis by phages, CO₂ requirement, H₂S production, dye (thionin and basic fuchsin) sensitivity and agglutination with monospecific A and

M antisera—did not reveal any differences between strain EP and *B. melitensis* strain 16M, the reference and type strain of biovar 1 of this species.

However, strain EP displayed simultaneously both smooth and rough characteristics. As any typical smooth strain of *B. melitensis* biovar 1, it was lysed by the Iz phage and was agglutinated by the monospecific M antiserum (table) but like a rough strain, it was lysed by the R/C phage and its colonies were stained by crystal violet and were agglutinated in a 1 in 1000 solution of neutral acriflavine and by the R specific serum (table).

The homogeneity of this unusual phenotype was tested on 10 randomly selected and cloned colonies, five on TSAYE medium and five on BAB medium. Whatever the medium, the 10 clones all had the typical “smooth-rough” properties described above.

To investigate these findings further, LPS of strain EP was analysed by biochemical and immunochemical methods.

Examination of homogeneity by FACS analysis

FACS analysis of strain EP showed that 99% of the population ($n = 50000$) bound to MAb A6812F12G12, specific for S-LPS, (mean signal = 58.5). In addition, MAb A6803F03D5, specific for R-LPS, stained 95% of the cells (mean signal = 23.15). In both cases, the fluorescence histograms (fig. 1) showed a narrow, unimodal distribution.

Fig. 3. Immunoblots of *B. melitensis* EP whole-cell lysate after SDS-PAGE on acrylamide 11% gels. Lane 1, molecular mass markers stained with Ponceau red after blotting; 2-7 correspond to *B. melitensis* EP whole-cell lysates; 8, purified R-LPS from *B. melitensis* B-115. Bands were detected by anti-M MAb (2), anti-A MAb (3), rabbit anti-M monospecific serum (4), anti-A monospecific serum (5), rabbit anti-RLPS monospecific serum (7) or by anti-R-LPS MAb (6 and 8). Material in lanes 3 and 5 were concentrated six-fold to improve the detection of the A antigen.

Fig. 4. Immunoblots after SDS-PAGE in acrylamide 11-35% gels of whole-cell lysates of *B. melitensis* EP (lanes 1 and 4); *B. melitensis* Rev-1 rough variant (2 and 5); purified R-LPS from rough *B. melitensis* B115 (3 and 6). Blots were detected by an anti-R-LPS MAb (1, 2 and 3) or by rabbit anti-R-LPS monospecific serum (4, 5 and 6).

Molecular analysis of LPS in SDS-PAGE

PK-LPS extracts analysed by SDS-PAGE in homogeneous acrylamide 11% gels revealed the pattern of M-predominating, ($M > A$) brucella S-LPS, found in biovar 1 of *B. melitensis* and characterised by fine bands with intervening larger and wider bands arranged in "triplets" (fig. 2). However, the intensity of staining was noticeably lower than when PK-LPS extracts prepared from the same estimated number of cells of *B. melitensis* 16M were used. On the other hand, a dark-stained, diffuse band corresponding to LPS lacking O-chains (R-LPS) was present at the bottom of the gel. S-LPS bands of strain EP were found at the same position as those of strain 16M, indicating an analogous distribution of mol. wt. In addition, some discrete bands were detected between the S-LPS and R-LPS zones.

When gradient gels of acrylamide 11-35% were used, the fast-migrating band was resolved as two well-separated bands, and the pattern of bands of S-LPS described as above was conserved.

Immunotype characterisation

Immunoblots obtained by transfer from homogeneous 11% gels clearly revealed the pattern already described for the silver-stained bands detected in the gels. This pattern was observed with rabbit hyper-immune monospecific anti-M sera and with anti-M MAb, thus eliminating the possibility of reaction against the core, i.e., the inner part of LPS (fig. 3).

Slide agglutination was not sufficiently sensitive to reveal the A antigens. However, the immunoblots from gels in which the amount of PK-LPS was increased six-fold confirmed the presence of A epitopes in S-LPS of strain EP (fig. 3).

Since immunoblots made from acrylamide 11-35% gradient gels increased the resolution for the fast migrating bands, these conditions were used to investigate the expression of R-LPS specificity. Anti-R-LPS monospecific serum detected two well separated bands in the area of low mol. wt (fig. 4). This was similar to those produced by R-LPS from *B. melitensis* strain B115 and by a PK lysate from *B. melitensis* strain Rev-1(R). The same bands were also revealed by anti-R MAb; S-LPS bands were not detected.

No differences were observed with this technique in analysing cell preparations derived from cultures grown on either BAB or TSAYE media, although the colony surfaces looked slightly different when observed directly.

Quantitative LPS determination

A quantitative analysis was used to test the relative amount of M-type S-LPS expressed. The agglutination-inhibition method allowed immunological assay of both complete and degraded forms of S-LPS.¹⁴ When PK-LPS samples obtained from *B.*

Fig. 5. Agglutination-inhibition curves of *B. melitensis* 16 M, (■) *B. melitensis* EP (□), and *B. melitensis* Rev1 (●) whole-cell lysates (●). Dilutions of lysates were incubated with anti-M MAb, S-LPS-coated latex was added, and the absorbances recorded and transformed into percentages of inhibition and plotted against PK-LPS dilution. Samples were at the same concentrations in terms of cellular dry-weight.

melitensis strain 16M were compared with similar samples from *B. melitensis* strain EP, the inhibition curves indicated that the latter contained as little as 10 times less S-LPS than the former (fig. 5). No inhibition was produced by *B. melitensis* strain Rev1(R) lacking O-chain which was included as a control. No qualitative differences were found with different culture media.

Discussion

Attention to the colonial morphology of a brucella culture is essential in the typing procedure, which is a key step in clinical microbiology. Non-smooth cultures cannot be typed with A and M monospecific sera nor with S phase-specific brucella phages. By direct observation, colonies of *B. melitensis* strain EP had a typical S phase appearance, confirmed by agglutination with anti-M serum and by lysis with phage Iz. However, the acriflavine test and staining of colonies with crystal violet unexpectedly revealed rough characteristics which were also shown by agglutination with anti-R serum and by lysis with the brucella phage R/C. This colonial morphology in a pure culture is a rare event; except for strain EP, it was never observed in more than 3000 *Brucella* strains typed at this laboratory (INRA-Nouzilly).

To exclude the possibility of a mixture of populations, strain EP was checked for purity by randomly cloning 10 colonies which all had a "smooth-rough" phenotype. In addition, we studied the homogeneity of strain EP by flow cytometry. In this context, FACS analysis offered the valuable advantage of determining the distribution of epitopes in a population, but on an individual cell basis, which could give information not available from mass, colonial-level studies. We screened for expression of S- and R-related epitopes. Both S- and R-specific MAbs bound specifically to strain EP. The distribution of fluorescent events was unimodal and narrow, revealing the homogeneous

expression of both parameters. Thus, FACS analysis confirmed that strain EP represents a homogeneous population and not a mixture of R and S cells.

The simultaneous expression of S- and R-LPS was also studied by SDS-PAGE. When compared with typical smooth and rough *Brucella* strains, the LPS pattern of strain EP (fig. 2) showed (i) an M-dominant structure, i.e., a succession of regularly spaced triplets as expected for a typical S phase strain of *B. melitensis* biovar 1,^{3,9} and (ii) two major fast-migrating bands characteristic of R-LPS, as resolved in blots from gradient gels. The distribution of S-LPS bands in the gel was the same for both strains EP and 16M, and was consistent with those observed previously.³ The bands between the S-LPS and R-LPS zones might correspond to protease-resistant outer-membrane proteins, a conclusion which is consistent with data reported previously.¹³

Immunoblots with anti-M and anti-R polyclonal antisera and MAbs confirmed the mixed "smooth-rough" structure of the LPS of strain EP by revealing the M-specificity of the triplets (fig. 3) and the R-specificity of the fast-migrating bands (fig. 4).

The simultaneous accessibility of the S and R epitopes of strain EP to their relevant antibodies and phages could be explained either by an unusual reduction of the number of full-size S-LPS chains at the surface of the bacteria, or by the expression of atypically short S-LPS chains. The absence of low mol. wt bands on immunoblots with anti-M antibodies (fig. 3) excludes the latter hypothesis and emphasises the former. Moreover, the expression at the surface of strain EP of fewer full-size S-LPS chains than in typical S phase *Brucella* strains was confirmed by a less intense silver staining of its S-LPS after SDS-PAGE (fig. 2) and by the much smaller amount of S-LPS indicated by quantitative analysis of PK-SDS extracts (fig. 5).

Recently, the use of cloned DNA probes has shown that strain EP (cited as BCCN 87.91) belongs to biovar 1 of *B. melitensis*, together with the reference *B.*

melitensis strain 16M.²² This confirmed the identity of strain EP as *B. melitensis* in spite of the atypical phage lysis results.

S phase brucella cultures tend to undergo variation in colonial morphology during growth. Henry (cited by Spink²³) identified six different colonial types including "intermediate" and "smooth-rough" self-agglutinating ones. Huddleson (cited by Spink²³) also described "smooth intermediate" (SI) and "mucoid" (M) colonies. Alton⁶ isolated *B. melitensis* strains from goats' milk which were found to be poor agglutinin inducers and less virulent for rabbits than typical S strains. By using agglutination and absorption tests, this author deduced that the antigenic structure of the strains could be viewed as "aRm", i.e., that R antigens were well exposed because A and M ones were present in very limited amounts. However, no explanation at a molecular level was given.

Our results, in agreement with those of Alton,⁶ can be related to similar findings in some *Salmonella* strains described by Naide *et al.*⁷ as "semi-rough" strains, i.e., behaving morphologically as S phase and serologically as R phase or *vice versa*. Palva and Mäkelä²⁴ reported a similar heterogeneity in a leaky Ra mutant of *S. typhimurium* that showed fewer S-LPS molecules of high mol. wt than current smooth isolates on SDS-PAGE. The particular phenotype of *Salmonella* rfak mutants,² very similar to that of strain EP, was found to be due to a reduced ability to attach O-chains because of the absence of an acetylglucosamine residue on carbon 2 of the glucose II unit of the LPS core.

As for the *Salmonella* "semi-rough" strains,^{7,25} the hypothesis of a simultaneous expression at the surface of the bacteria of relatively few full-size O-chains but numerous R-LPS "stubs" is in agreement with our SDS-PAGE, immunoblotting and quantitative results and could, therefore, account for the morphological phenotype of strain EP. The fact that full-size S-LPS molecules are expressed at a low level probably means that all the biosynthetic steps are operative but that one is limiting, either in the polymerisation of O-chain or during the translocation of the O-chain to the core. Information is not currently available on brucella LPS biosynthesis.

Major changes in phase are relatively easy to detect in brucella cultures by the standard bacteriological methods. However, when minor variations are present in a strain, biochemical and immunochemical analysis of LPS are necessary, as demonstrated. As these changes involve both qualitative and quantitative variations of relevant epitopes, this approach can be useful in the typing of new clinical isolates, such as the EP strain, as well as in the control of reference and vaccine strains of *Brucella*.

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