Growth of calcium-blind mutants of *Yersinia pestis* at 37 °C in permissive Ca^{2+} -deficient environments

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Cells of wild-type *Yersinia pestis* exhibit a low-calcium response (LCR) defined as bacteriostasis with expression of a pCD-encoded type III secretion system (T3SS) during cultivation at 37 °C without added Ca^{2+} versus vegetative growth with downregulation of the T3SS with Ca^{2+} (≥ 2.5 mM). Bacteriostasis is known to reflect cumulative toxicity of Na^+ , L-glutamic acid and culture pH; control of these variables enables full-scale growth ('rescue') in the absence of Ca^{2+} . Several T3SS regulatory proteins modulate the LCR, because their absence promotes a Ca^{2+} -blind phenotype in which growth at 37 °C ceases and the T3SS is constitutive even with added Ca^{2+} . This study analysed the connection between the LCR and Ca^{2+} by determining the response of selected Ca^{2+} -blind mutants grown in Ca^{2+} -deficient rescue media containing Na^+ plus L-glutamate (pH 5.5), where the T3SS is not expressed, L-glutamate alone (pH 6.5), where L-aspartate is fully catabolized, and Na^+ alone (pH 9.0), where the electrogenic sodium pump NADH : ubiquinone oxidoreductase becomes activated. All three conditions supported essentially full-scale Ca^{2+} -independent growth at 37 °C of wild-type *Y. pestis* as well as *lcrG* and *yopN* mutants (possessing a complete but dysregulated T3SS), indicating that bacteriostasis reflects a Na^+ -dependent lesion in bioenergetics. In contrast, mutants lacking the negative regulator YopD or the YopD chaperone LcrH failed to grow in any rescue medium and are therefore truly temperature-sensitive. The Ca^{2+} -blind *yopD* phenotype was fully suppressed in a Ca^{2+} -independent background lacking the injectisome-associated inner-membrane component YscV but not peripheral YscK, suggesting that the core translocon energizes YopD.

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INTRODUCTION

The medically significant yersiniae are *Yersinia pestis*, the causative agent of bubonic plague, the very closely related *Yersinia pseudotuberculosis*, and the more divergent *Yersinia enterocolitica* (Achtman *et al.*, 1999; Moore & Brubaker, 1975). All three species share a similar ~70 kb plasmid, termed pCD in *Y. pestis* and pYV in enteropathogenic *Y. pseudotuberculosis* and *Y. enterocolitica*. This element mediates a low-calcium response (LCR) that is expressed at 37 °C but not 26 °C and defined as the cessation of vegetative growth and expression of a pCD/pYV-encoded type III secretion system (T3SS) during cultivation without Ca^{2+} as opposed to vegetative growth accompanied by downregulation of this T3SS in the presence of ≥ 2.5 mM

Ca^{2+} (Brubaker & Surgalla, 1964; Higuchi *et al.*, 1959; Kupferberg & Higuchi, 1958; Portnoy *et al.*, 1981; Straley & Brubaker, 1981) (Lcr^+). Temperature-controlled upregulation of the T3SS occurs by thermal release of YmoA-imposed downregulation (Cornelis *et al.*, 1991) of a pCD/pYV-encoded transcriptional activator termed LcrF/VirF (Cornelis *et al.*, 1989; Hoe & Goguen, 1993), whereas downregulation by Ca^{2+} involves the negative regulator YopD and its chaperone LcrH/SycD (Francis *et al.*, 2001; Williams & Straley, 1998), as well as T3SS 'gate' complexes YopN–SycN–YscB–TyeA (Cheng *et al.*, 2001; Ferracci *et al.*, 2005) and LcrG–LcrV (Matson & Nilles, 2001; Nilles *et al.*, 1997), which prevent access of virulence effectors to the translocon. Contact of yersiniae with the host cell surface, even in Ca^{2+} -rich mammalian plasma or lymph (Kugelmass, 1959), abrogates this inhibition (Cornelis & Wolf-Watz, 1997; Rosqvist *et al.*, 1994), allowing the translocation of T3SS virulence effectors termed Yops into

Abbreviations: Caf1, capsular antigen fraction 1; LCR, low-calcium response; T3SS, type III secretion system.

the host cell. This relationship suggests that competition occurs at the bacterial surface between Ca^{2+} and a host receptor, although the nature of the steric changes enabling this distinction is not fully resolved. However, Ca^{2+} -starved Lcr^+ (pCD+) yersiniae bind more carrier-free $^{45}\text{Ca}^{2+}$ at 37 °C than do Lcr^- (pCD-) mutants (Perry & Brubaker, 1987), and the results of recent studies suggest that YscF, a small protein that polymerizes to form the external needle of the T3SS injectisome, is essential for calcium-dependent regulation (Torruellas *et al.*, 2005).

Less is known about the basis of the temperature-dependent nutritional requirement of Lcr^+ yersiniae for Ca^{2+} . Early findings showed that Mg^{2+} (Brubaker & Surgalla, 1964; Higuchi & Smith, 1961) and Na^+ (Brubaker, 1967) augment the requirement for Ca^{2+} , which could be largely replaced by Sr^{2+} , Zn^{2+} (Higuchi *et al.*, 1959) or exogenous nucleoside triphosphates at mildly alkaline pH (Zahorchak & Brubaker, 1982). Ca^{2+} -starved Lcr^+ cells of *Y. pestis* probably possess a lesion in energy metabolism, as judged by their large size (Brubaker & Surgalla, 1964), presence of axial filaments (Hall *et al.*, 1974) and reduced adenylate energy charge (Zahorchak *et al.*, 1979) typical of late stationary phase. Bacteriostasis of Lcr^+ cells of *Y. pestis* following shift to 37 °C in Ca^{2+} -deficient medium is abrupt, as opposed to the situation in enteropathogenic yersiniae, which typically undergo three or more additional doublings (*Y. pseudotuberculosis*) or multiply indefinitely at a reduced rate (*Y. enterocolitica*) (Brubaker, 2005, 2007; Carter *et al.*, 1980). This difference is thought to reflect mutational loss of the enzyme aspartase (AspA) in *Y. pestis*, which results in excretion of L-aspartic acid by Ca^{2+} -starved cells at the expense of exogenous L-glutamic acid (Brubaker, 2005; Dreyfus & Brubaker, 1978). Na^+ enhances this effect but permits full-scale Ca^{2+} -independent growth at alkaline pH (9.0). Other permissive or 'rescue' Ca^{2+} -deficient environments are low pH (5.5), where LcrF/VirF -activated functions are not detected, and cytoplasmic pH (6.5) with L-glutamate in the absence of Na^+ , where metabolic L-aspartate is entirely catabolized and pCD-encoded virulence factors are fully expressed (Brubaker, 2005, 2007).

Two types of mutants exhibiting altered responses to Ca^{2+} have been instrumental in defining the regulation and physiology of the LCR. The first are Ca^{2+} -independent isolates characterized by growth at 37 °C (with or without Ca^{2+}) with inability to express LcrF/VirF -activated determinants; the second are Ca^{2+} -blind mutants that constitutively express these factors at 37 °C but are unable to grow even if Ca^{2+} is provided (Goguen *et al.*, 1984). In this report, we determined the effects of the three Ca^{2+} -deficient rescue conditions on the phenotypes of some well-characterized Ca^{2+} -independent and Ca^{2+} -blind mutants. The findings supported the hypothesis that Ca^{2+} -dependent growth of wild-type *Y. pestis* at host temperature *in vitro* reflects Na^+ -sensitivity imposed by operation of the T3SS. The data also revealed that Ca^{2+} -blind *lcrG* or *yopN* but not *yopD* or *lcrH* isolates could be

rescued by control of pH, Na^+ and L-glutamate, and that constitutive bacteriostasis of the last two mutants could be suppressed by removal of essential inner core T3SS function (*yscV*) as opposed to repair of the gateways mediated by *LcrG* and *YopN*.

METHODS

Bacteria. All strains of *Y. pestis* used in this study are derivatives of KIM-5 (Finegold *et al.*, 1968). Sources and characteristics of these isolates are given in Table 1. Storage of bacteria at -20 °C in buffered glycerol and their recovery on solid medium for use in experiments has been previously described (Beesley *et al.*, 1967). Carriage of pCD in populations of Ca^{2+} -dependent or Ca^{2+} -blind strains was routinely monitored by plating on magnesium oxalate agar (Higuchi & Smith, 1961), and its presence in Ca^{2+} -independent *lcrV* and *yscV* mutants was determined by electrophoresis in agarose as previously described (Ferber & Brubaker, 1981). Retention of pPCP was monitored by expression of pesticin on blood agar base (Difco) using *Y. pseudotuberculosis* strain PB1 as an indicator (Brubaker & Surgalla, 1962). Mutants cured of pPCP were selected directly on this medium after enrichment by passage at 5 °C (Sample *et al.*, 1987).

yscK was deleted from the virulence plasmid via allelic exchange, resulting in *Y. pestis* KIM8-3002.12. pAW211 for deletion of *yscK* was constructed as follows. Primers YscI313-A (5' CGGAATTCAGCCAA-AATGTTGAAACCTTGTCG 3') and YscKΔKpn-B (5' GACAAGT-TTGTGACATAAAGTTTGGGTACCCATCACTTCT TGCGTTGTA-ACCAA 3') were used to amplify a 776 bp region upstream of *yscK* and introduce unique *EcoRI* and *KpnI* restriction sites. Primers YscKΔKpn-A (5' TGGGTTACAACGCAAGAAGTGATGGGTAC-CCAACTTTATGTCACAACTTGTC 3'), complementary to YscKΔKpn-B, and YscL+63-B (5' CGGGATCCCCAATTTAGGTA-GATAACTCAATC 3') were used to amplify a 674 bp region downstream of *yscK* and introduce unique *KpnI* and *BamHI* restriction sites. The two PCR products were purified and digested with *EcoRI/KpnI* and *KpnI/BamHI*, respectively, and the digested fragments were then ligated into *EcoRI*- and *BamHI*-cut pLD55. Because *yscL* overlaps *yscK*, the design of the YscKΔKpn primers retained the native *yscL* translational initiation codon (for a total of 65 bp of the 3' end of *yscK*) and an additional 10 bp of upstream *yscK*. Plasmid pAW211 was propagated in *Escherichia coli* SY327 (*Δpir*). Deletion vector pAW211 was used to delete *yscK* from the LCR plasmid (pCD1) by allelic exchange, as described previously (Metcalf *et al.*, 1996; Nilles *et al.*, 1998). PCR as well as immunoblot analysis confirmed tetracycline-sensitive clones for gene deletion.

The non-polarity of the resulting *yscK* mutation was demonstrated by restoration of the wild-type LCR when YscK with a C-terminal haemagglutinin epitope was expressed *in trans* from the arabinose-inducible promoter in pYscKHA18. To make this plasmid, the 630 bp *yscK* gene was amplified with primers YscK1SD-A (5' GCTAG-AGCTCAGGAGGAACATATGGAAAATTATATTACCTCT 3') and YscK630HA-B (5' GATCTAGATTAAGCATAATCTGGAACATCA-TATGGATATTTGAACAAA 3'). The forward primer created an artificial Shine-Dalgarno sequence and a unique *SacI* restriction site, while the reverse primer introduced the haemagglutinin epitope onto the C terminus of YscK as well as a unique *XbaI* restriction site. The resultant PCR product was purified, digested with *SacI* and *XbaI*, and ligated into pBAD18-Km^r (Guzman *et al.*, 1995) that had been digested with *SacI* and *XbaI*. The resulting pYscKHA18 plasmid was electroporated into the ΔyscK *Y. pestis* KIM8-3002.12.

To make the complementation test, the parent YscK⁺ *Y. pestis* KIM8-3002, *Y. pestis* KIM8-3002.1.2 and *Y. pestis* KIM8-3002.2.2 were grown for approximately seven generations at 26 °C in the defined

Table 1. Bacterial strains used in this study

Strain	Description*†	Source or reference
<i>Y. pestis</i> strains		
KIM5	pCD1, pPCP1, pMT1; Lcr ⁺ ; Pgm ⁻ wild-type parental strain; also called D27	R. R. Brubaker
KIM6	Lcr ⁻ (pCD1 ⁻), pPCP1, pMT1; also called D28	R. R. Brubaker
KIM8-3002	Sm ^R ; pCD1 (Lcr ⁺), Pla ⁻ (pPCP1 ⁻), pMT1	Nilles <i>et al.</i> (1998); unpublished data
KIM8-3002.7	Sm ^R ; pCD1 (Δ lcrG3 [6–86]), Pla ⁻ (pPCP1 ⁻), pMT1	Fields <i>et al.</i> (1999)
KIM5-3001.6	Sm ^R ; pCD1 (Δ yopN [48–197]), pPCP1, pMT1	Plano & Straley (1995)
KIM5-3401	Sm ^R ; pCD1 (<i>lcrH</i> :: <i>cat</i> , <i>yopJ</i> ::MudI1734 [Km ^R Lac ⁺][YscC ⁻ LcrH ⁻ YopB ⁻ YopD ⁻ YopJ ⁻]), pPCP1, pMT1	Price & Straley (1989)
KIM5-3241.2	Sm ^R Km ^R Lac ⁺ ; pCD1 (Δ lcrV [18–215, <i>yopJ</i> ::MudI1734]), pPCP1, pMT1	Price <i>et al.</i> (1991)
KIM8-3002.2	Sm ^R ; pCD1(Δ yopD [1–305]), Pla ⁻ (pPCP1 ⁻), pMT1	Williams & Straley (1998)
KIM5-3001.2.1	Sm ^R ; pCD1 (Δ yscV [192–343] Δ yopD [1–305]), pPCP1, pMT1	Williams & Straley (1998)
KIM8-3002.12	Sm ^R ; pCD1 (Δ yscK [4–190]), Pla ⁻ (pPCP1 ⁻), pMT1	This study
KIM8-3002.2.2	Sm ^R ; pCD1 (Δ yopD [1–305]), Δ yscK (4–190) Pla ⁻ (pPCP1 ⁻), pMT1	This study
<i>E. coli</i> strain		
SY327(λ pir)	Δ (<i>lac-pro</i>) <i>argE</i> (Am) <i>rif</i> <i>nalA</i> <i>recA</i> 56 λ pir	Miller & Mekalanos (1988)

*Numbers within the gene designation parentheses represent the amino acids deleted from the encoded protein.

†All *Y. pestis* strains are Pgm⁻. Native plasmids of *Y. pestis* are the LCR plasmid pCD1, the Pla-encoding pPCP1 and pMT1 (Ferber & Brubaker, 1981), which encodes Caf1 (Protsenko *et al.*, 1983).

medium TMH containing or lacking 2.5 mM CaCl₂ (Straley & Bowmer, 1986), the temperature was shifted to 37 °C, 0.2 % arabinose was added to the cultures, and growth was continued another 4 h. Yop expression and secretion were determined via immunoblot analysis as previously described (Wulff-Strobel *et al.*, 2002). As is typical for *ysc* mutants, the *yscK* mutation caused downregulated expression and blocked secretion of Yops (illustrated in Fig. 1, top panel, for YopM and LcrV). Other experiments evaluated expression and secretion of YopD and LcrQ with similar results (data not shown). Further, expression of YopM from the multicopy plasmid pTrcM.2 (Plano & Straley, 1993) resulted in high levels of intracellular YopM, which was not secreted (data not shown). In experiments in which the incubation at 37 °C was continued for 7–8 h, the LCR growth phenotype was demonstrated: the Δ yscK bacteria failed to show growth restriction, in contrast to the parent *Y. pestis* KIM8-3002 and the complemented mutant (data not shown). The deletion mutation appeared to be non-polar, in that it could be complemented for both Yop expression and secretion when YscK fused to the haemagglutinin epitope was expressed from pYscKHA-18 (Fig. 1, bottom panel).

Media and cultivation. The basic chemically defined medium used in all other experiments has been described previously (Brubaker, 2005; Fowler & Brubaker, 1994). Precise instructions for preparing and reconstituting the eight stock solutions composing this medium are available (Brubaker, 2007); the freshly prepared or stored salts stock solution was warmed prior to use to assure full solubility of MgO. Permissive Ca²⁺-deficient versions used in the present study contained Na⁺ (100 mM) plus L-glutamic acid (25 mM) initially buffered at pH 5.5 with 25 mM MES, L-glutamic acid (25 mM) without added Na⁺ initially buffered at pH 6.5 with 25 mM MOPS, or Na⁺ (100 mM) without added L-glutamic acid initially buffered at pH 9.0 with 25 mM CHES. Also included as a control was the highly restrictive Ca²⁺-deficient combination of Na⁺ (100 mM) plus L-glutamic acid (25 mM) initially buffered at pH 7.5 with 25 mM TRIS. First and second transfers prepared to inoculate third cultures for use in experiments were always initially buffered at pH 7.0 with 25 mM HEPES. Parallel determinations were also undertaken in control cultures containing 4.0 mM Ca²⁺ (present only during the third

transfer). Rates of growth were determined in Erlenmeyer flasks (25 ml medium per 250 ml flask) aerated at 200 r.p.m. in a temperature-controlled model G76 water bath gyrotory shaker (New Brunswick Scientific). First and second transfers were inoculated at OD₆₂₀ ~0.1, incubated at 26 °C, and transferred during the late exponential growth phase at OD₆₂₀ 5–6. Third cultures comprising the four cultural conditions noted above were then inoculated at OD₆₂₀ ~0.12 and incubated at 37 °C. Bacterial growth was determined at intervals with a Beckman DU spectrophotometer using 1 ml cuvettes containing samples suitably diluted in 0.033 M potassium phosphate buffer (pH 7.0) to yield an OD₆₂₀ between 0.05 and 0.7.

Assay of LcrV and Yops secretion. Samples of parallel final (third transfer) cultures were removed after cultivation for 7 h, and total Yops and LcrV were analysed in TCA-precipitated culture supernatants by SDS-PAGE followed by silver staining or immunoblotting, performed as previously described (Brubaker *et al.*, 1987; Motin *et al.*, 1994). Positions of individual Yops and LcrV on stained SDS-PAGE gels were assigned according to known molecular masses (Hu *et al.*, 1998) and specific control antisera.

RESULTS

Growth phenotypes of parental and mutant *Y. pestis* under permissive and stringent conditions

In this study, we tested whether Ca²⁺-blind *Y. pestis* mutants have LCRs that are equivalent to that of the parental strain by comparing growth and expression of Yops and LcrV in a restrictive medium and three rescue media. When Ca²⁺ was present, all four tested environments supported full-scale growth of parental Lcr⁺ yersiniae as well as Ca²⁺-independent Lcr⁻ and LcrV⁻ mutants (Fig. 2). In contrast, abrupt shut-off of LcrG⁻ mutants occurred at 8 h with added Na⁺ and L-glutamate

Fig. 1. Complementation of the $\Delta yscK$ *Y. pestis* mutant used in this work. Non-polarity of the deletion within *yscK* in *Y. pestis* KIM8-3002.12 was tested by comparing the LCR phenotypes of the parental *Y. pestis* KIM8-3002, $\Delta yscK$ *Y. pestis* KIM8-3002.12 and the *Y. pestis* KIM8-3002.12 pYscKHA18 ($YscK^+$) isolates. The bacteria were grown in TMH with (+) or without (-) 2.5 mM $CaCl_2$ for 4 h at 37 °C, and YopM and LcrV abundance was assessed by immunoblotting in the bacterial cells (c) or the medium containing secreted proteins (s). Top panel, the *yscK* deletion mutant was compared for its Yops expression and secretion (lanes 5–8) with the parental strain (lanes 1–4); bottom panel, complementation by the haemagglutinin-tagged YscK ($YscK$ -HA) expressed from pYscKHA18 was assessed by comparing Yops expression and secretion of the complemented strain (lanes 5–8) with those of the *yscK* mutant (lanes 1–4).

at pH 7.5, demonstrating the Ca^{2+} -blind phenotype in this environment (Fig. 2a). Multiplication of $LcrG^-$ cells continued for 10 h with added L-glutamate (no added Na^+) at pH 6.5 (Fig. 2b), at which time the pH approached neutrality, causing growth to slow because the condition now resembled a milder version of that shown in Fig. 2(a). Full-scale growth occurred with added Na^+ (no added L-glutamate) at pH 9.0 (Fig. 2c) or added Na^+ and L-glutamate at pH 5.5 (Fig. 2d). A similar pattern was observed for $YopN^-$ mutants. However, growth of $YopD^-$ mutants was restricted under all four conditions, with the strongest response occurring at pH 7.5 with added Na^+ and L-glutamate. The progressive decrease of optical density of the $YopD^-$ *Y. pestis* culture at pH 9.0 with added Na^+ and Ca^{2+} (less L-glutamate) probably reflected aggregation with precipitated calcium phosphate rather than cell lysis (Fig. 2b). The $LcrH^-$ mutant showed a similar, but not identical, phenotype to that of the $YopD^-$ strain, consistent with the requirement of LcrH for stability of YopD (Wattiau *et al.*, 1994) combined with a direct regulatory effect of LcrH on the T3SS (Francis *et al.*, 2001).

In summary, in the absence of added Ca^{2+} only Ca^{2+} -independent Lcr^- and $LcrV^-$ *yersiniae* exhibited full-scale growth in the restrictive environment of pH 7.5 with Na^+ and L-glutamate, as expected (Fig. 3a). However, these two isolates, parental Lcr^+ cells, and Ca^{2+} -blind $LcrG^-$ and $YopN^-$ strains all showed Ca^{2+} -independent growth in the three permissive media (Fig. 3b–d). In contrast, multiplication of $YopD^-$ and $LcrH^-$ mutants was sometimes improved in the rescue media, although these environments failed to promote full-scale growth.

Secretion of Yops by parent and mutant strains

As shown by SDS-PAGE, none of the Ca^{2+} -blind mutants could downregulate expression of Yops in the restrictive environment (pH 7.5 with Na^+ and L-glutamate) containing added Ca^{2+} (Fig. 4a, lanes 4, 6, 7 and 8) as did the Lcr^+ parent (Fig. 4b, lanes 2 and 3). These findings verify that the tested Ca^{2+} -blind mutants are unable to utilize Ca^{2+} for downregulation of Yops and LcrV. Nevertheless, growth at 37 °C of the $LcrG^-$ and $YopN^-$ isolates cultivated in the rescue medium containing 100 mM Na^+ without L-glutamate (pH 9.0) (Fig. 5) or no added Na^+ with 25 mM L-glutamate (pH 6.5) (Fig. 6) was accompanied by significant expression of Yops and LcrV. In contrast, growth at pH 5.5 with both 100 mM Na^+ and 25 mM L-glutamate barely permitted detection of these effectors, as noted previously; this effect is emphasized by including the corresponding silver-stained gels (Fig. 7). In contrast, these three rescue media were unable to promote multiplication of $LcrH^-$ and $YopD^-$ mutants, which therefore appear to be truly temperature-sensitive.

Suppression of the LCR in $YopD$ isolates

Attempts to promote growth of the $YopD^-$ mutant at 37 °C by addition of nucleoside triphosphates or polyamines, or replacement of Ca^{2+} with Sr^{2+} or Zn^{2+} , were not successful (data not shown). We then tested whether a mutation in the T3SS that abolishes secretion and causes Ca^{2+} -independent growth would exert intergenic suppression on the *yopD* phenotype. A *yscK yopD* double mutant still ceased growth promptly, regardless of the presence of Ca^{2+} , whereas a *yscV yopD* mutant was no longer temperature-sensitive (Fig. 8). As expected, the *yscK* and *yscV* mutations abolished T3SS secretion, whether present alone or in combination with the *yopD* mutation (data not shown), but the two double mutants had strikingly different levels of Yops and LcrV production (Fig. 9). In keeping with the LCR growth phenotypes of these double mutants, the *yscK yopD* mutant still expressed abundant Yops and LcrV, whereas the *yscV yopD* mutant was unable to express significant amounts of these virulence factors. These findings showed that it is possible to override the *yopD* phenotype by a T3SS mutation and that this phenomenon is independent of blocking secretion per se.

Fig. 2. Growth of Lcr^+ (●), Lcr^- (○), $LcrG^-$ (▲), $LcrV^-$ (△), $LcrH^-$ (▼), $YopD^-$ (◆) and $YopN^-$ (■) mutants of *Y. pestis* strain KIM in chemically defined medium containing 4.0 mM Ca^{2+} with (a) 100 mM Na^+ and 25 mM L-glutamic acid (pH 7.5), (b) no added Na^+ and 25 mM L-glutamic acid (pH 6.5), (c) 100 mM Na^+ and no added L-glutamic acid (pH 9.0), and (d) 100 mM Na^+ and 25 mM L-glutamic acid (pH 5.5). The dashed line indicates agglutination of *yopD* cells with precipitated calcium phosphate at pH 9.0.

DISCUSSION

Major variables now known to modulate the LCR of *Y. pestis* include carriage of pCD, temperature, Ca^{2+} , Na^+ , the naturally occurring dicarboxylic acids, pH and AspA. While the nature of the interactions among these variables is not fully resolved, it is clear that Ca^{2+} , culture at room temperature, and low pH cause downregulation of the pCD-encoded T3SS. Results of recent work show that bacteriostasis of wild-type yersiniae associated with Ca^{2+} -deprivation at 37 °C can be entirely prevented at the mildly acidic pH of cytoplasm by eliminating Na^+ from the medium and supplying L-glutamic acid (Brubaker, 2005, 2007). The latter is a favoured energy source of *Y. pestis* (Higuchi *et al.*, 1959) due to its immediate access to the tricarboxylic acid cycle, assuring prompt and complete catabolism. Yersiniae growing in this permissive Ca^{2+} -deficient environment expressed LcrV and a full complement of Yops. These findings show that upregulation of Yops and LcrV synthesis as well as steady-state growth can occur together *in vitro* and suggest that Na^+ is only toxic to *Y. pestis* when the T3SS is operating. The present study tested this prediction and identified a hierarchy of T3SS components that modulate the response to Ca^{2+} -deprivation. Further resolution of these interactions will entail

defining the physiological process that is inhibited by Na^+ and the mechanism that permits Na^+ to access this likely cytoplasmic target.

If Ca^{2+} indeed causes downregulation of a T3SS-mediated process that leads to Na^+ sensitivity, then Ca^{2+} -blind mutants should be able to grow at 37 °C in the absence of Na^+ , whether Ca^{2+} is present or not. This was the case for the *lcrG* and *yopN* mutants in the first permissive Ca^{2+} -deficient environment (no added Na^+ , 25 mM L-glutamate, pH 6.5; Figs 2b and 3b). This observation illustrates that constitutive expression of Yops at 37 °C caused by inability to form functional YopN–SycN–YscB–TyeA (Cheng *et al.*, 2001; Ferracci *et al.*, 2005) and LcrG–LcrV (Matson & Nilles, 2001; Nilles *et al.*, 1997) complexes is not inimical to vegetative growth provided that Na^+ is absent. These *yopN* and *lcrG* mutants possess otherwise fully functional injectisomes and thus suffer constitutively from the same putative Na^+ -dependent bioenergetic lesion that affects Lcr^+ cells in Ca^{2+} -deficient environments. In contrast, the absence of YopD or the instability of YopD due to the absence of LcrH, its cognate chaperone, had a distinct effect (as discussed below), and mutants lacking YopD or LcrH were not rescued by this cytoplasm-mimicking environment.

Fig. 3. Growth of Lcr^+ (●), Lcr^- (○), $LcrG^-$ (▲), $LcrV^-$ (△), $LcrH^-$ (▼), $YopD^-$ (◆) and $YopN^-$ (■) mutants of *Y. pestis* strain KIM in chemically defined medium lacking added Ca^{2+} with (a) 100 mM Na^+ and 25 mM L-glutamic acid (pH 7.5), (b) no added Na^+ and 25 mM L-glutamic acid (pH 6.5), (c) 100 mM Na^+ and no added L-glutamic acid (pH 9.0), and (d) 100 mM Na^+ and 25 mM L-glutamic acid (pH 5.5).

The second permissive Ca^{2+} -deficient environment (100 mM Na^+ , no added L-glutamate, pH 9.0) also supported full-scale growth of *lcrG* and *yopN* but not *lcrH* or *yopD* mutants. The reason why this medium accommodates growth with expression of the T3SS and attendant virulence effectors is not resolved but it is probably significant that NADH:ubiquinone oxidoreductase, known to exist in yersiniae (Chain *et al.*, 2004; Deng *et al.*, 2002; Parkhill *et al.*, 2001), can function under these conditions (Zhou *et al.*, 1999). As a consequence, accumulation of cytoplasmic Na^+ would be prevented via an electrogenic process that might also prevent the putative bioenergetic lesion believed to account for bacteriostasis. The third permissive condition (100 mM Na^+ , 25 mM L-glutamate, pH 5.5) also promoted multiplication of *lcrG* and *yopN* but not *yopD* mutants, although some residual growth of the *lcrH* isolate was observed. This environment was chosen because it prevents expression of the T3SS by Lcr^+ cells, as illustrated for Yops and *LcrV* in Fig. 7, and thus fails to generate the putative associated lesion that elicits bacteriostasis in the presence of Na^+ . Considered together, these results indicate that the same impairment that prevents Lcr^+ organisms from growing at 37 °C in the absence of Ca^{2+} occurs constitutively in Ca^{2+} -blind *lcrG* and *yopN* mutants, whereas *lcrH* and *yopD* mutants possess a distinct, true, temperature-dependent lesion.

Further study showed that this lesion was suppressed when the *yopD* strain also carried a deletion within *yscV* that rendered the encoded YscV non-functional; this effect was not seen in a *yscK yopD* double mutant, even though both the *yscV* and the *yscK* mutation abolished secretion and caused Ca^{2+} -independent growth when present alone (Figs 8 and 9). Previously, it has been observed that inactivation of *yscC* or *yscD* suppressed Ca^{2+} -blind growth and the Yops expression phenotypes due to mutations in *lcrG* and *yopN*. The double mutants also exhibited Ca^{2+} -independent growth and downregulated Yops expression, similar to strains having only the single *yscC* or *yscD* mutations (Plano & Straley, 1995). However, these secretion-blocking *ysc* mutations did not suppress the Ca^{2+} -blind LCR phenotype of an *lcrH* mutant, in which YopD is destabilized. Further, a mutant carrying an in-frame internal deletion within *yscR* is Ca^{2+} -independent in growth and shows downregulated Yops expression and blocked secretion of Yops (Fields *et al.*, 1994); but this mutation also did not suppress the Ca^{2+} -blind LCR phenotype of a *yopD* deletion (A. W. Williams & S. C. Straley, unpublished data). YscV is a core inner-membrane component of the type III translocons of numerous bacterial species, including the putative ancestral flagellar translocon (Plano & Straley, 1993; Plano *et al.*, 1991). It is predicted to have eight N-terminal transmembrane

Fig. 4. Immunoblots (YopH, YopD and LcrV) and silver-stained preparations following SDS-PAGE of whole cultures of Lcr⁻, Pla⁻ (lane 1); Lcr⁺, Pla⁺ (lane 2); Lcr⁺, Pla⁻ (lane 3); *lcrG*, Pla⁻ (lane 4); *lcrV*, Pla⁻ (lane 5); *lcrH*, Pla⁻ (lane 6); *yopD*, Pla⁻ (lane 7); and *yopN*, Pla⁻ (lane 8) cells of *Y. pestis* strain KIM after 7 h incubation at 37 °C in chemically defined medium with 100 mM Na⁺ and 25 mM L-glutamic acid (pH 7.5) either containing (a) or lacking (b) added 4.0 mM Ca²⁺; also shown in both panels are Ca²⁺-deficient control cultures of Lcr⁺, Pla⁻ cells following growth for 9 h in the same Ca²⁺-deficient medium (lane 9). The amount of the ~19 kDa band corresponding to fibrillar capsular antigen fraction 1 (Caf1) (open arrows) varied in amount among the strains and correlated with their net growth following shift from 26 to 37 °C.

domains and a large cytoplasmic C-terminal domain (McMurry *et al.*, 2004; Plano & Straley, 1993). The *yscV* mutation employed in this study would remove the last four of the transmembrane domains without removing the largest cytoplasmic loop domain (which in the mutant would be fused to the C-terminal cytoplasmic domain). This mutant produces a stable but non-functional YscV protein (Plano & Straley, 1993). The flagellar orthologue of YscV, FlhA, is a central component of the translocon: it interacts with other conserved integral membrane elements of the flagellar translocon (FliO, FliP and FliQ), and its cytoplasmic domain functions as a docking point for substrates onto the translocon by interacting with a

conserved component (FlhB) that regulates the type of substrate that has access to the translocon as well as the peripheral chaperone-ATPase-substrate complex (FliJ, FliI and FliH) (McMurry *et al.*, 2004; Minamino & Macnab, 2000). YscR shares similarity with the conserved flagellar translocon element FliP and notably has a large predicted cytoplasmic domain (Fields *et al.*, 1994), raising the possibility that it also interacts with YscV through this domain. The *yscV* mutation used in our work was chosen so as potentially to retain such peripheral interactions with substrates and translocon regulators, while being defective in a core translocon mechanism. In contrast to YscV, the T3SS components YscC, YscD and YscK all represent

Fig. 5. Immunoblots (YopH, YopD and LcrV) of whole Pla^- cultures of Lcr^+ (lane 1), *lcrG* (lane 2), *yopN* (lane 3), *lcrH* (lane 4) and *yopD* (lane 5) yersiniae after 7 h incubation at 37 °C in chemically defined medium containing 100 mM Na^+ and no added L-glutamic acid (pH 9.0) either containing (a) or lacking (b) added 4.0 mM Ca^{2+} ; also shown in both panels are Ca^{2+} -deficient control cultures of Lcr^+ , Pla^- cells following growth in the defined medium with added 100 mM Na^+ and 25 mM L-glutamate (pH 7.5) for 9 h (lane 6).

Fig. 6. Immunoblots (YopH, YopD and LcrV) of whole Pla^- cultures of Lcr^+ (lane 1), *lcrG* (lane 2), *yopN* (lane 3), *lcrH* (lane 4) and *yopD* (lane 5) yersiniae after 7 h incubation at 37 °C in chemically defined medium containing no added Na^+ and 25 mM L-glutamic acid (pH 6.5) either containing (a) or lacking (b) added 4.0 mM Ca^{2+} ; also shown in both panels are Ca^{2+} -deficient control cultures of Lcr^+ , Pla^- cells following growth in the defined medium with added 100 mM Na^+ and 25 mM L-glutamate (pH 7.5) for 9 h (lane 6).

Fig. 7. Immunoblots (YopH, YopD and LcrV) and silver-stained preparations following SDS-PAGE of whole Pla^- cultures of Lcr^+ (lane 1), lcrG (lane 2), yopN (lane 3), lcrH (lane 4) and yopD (lane 5) after 7 h incubation at 37 °C in chemically defined medium containing 100 mM Na^+ and 25 mM L-glutamate (pH 5.5) either containing (a) or lacking (b) added 4.0 mM Ca^{2+} ; also shown in both panels are Ca^{2+} -deficient control cultures of Lcr^+ , Pla^- cells following growth in the defined medium with added 100 mM Na^+ and 25 mM L-glutamate for 9 h (lane 6).

peripheral elements that evolved to adapt the core inner-membrane translocon to a virulence-associated role and are absent from the flagellar system or so extensively altered as to lack significant sequence similarity (Medini *et al.*, 2006). Nonetheless, if any of these T3SS components is missing, secretion is completely blocked, whether the block occurs due to formation of abnormal peripheral complexes (due to loss of YscK) or to altered inner-membrane or

outer-membrane architecture of the secretion machine (due to loss of YscD or YscC, respectively). It is thus not surprising that, due to such derangements of the secretion machine, the Ca^{2+} -independent LCR phenotype is dominant over Na^+ -sensitivity due to loss of 'gate proteins' such as YopN and LcrG that promote activation of the T3SS. This dominance occurs because the T3SS is permanently inactive and cannot cause the hypothetical

Fig. 8. Suppression of constitutive Ca^{2+} dependence in YopD⁻ mutants of *Y. pestis* KIM by *yscV* but not *yscK* mutations. Patterns of growth are shown for control Lcr⁺, Pla⁺ (●); Lcr⁻, Pla⁺ (○); Lcr⁺, Pla⁻ (▲); *yscV* (△); *yscK* (▼); *yopD* (■); *yopD yscV* (□); and *yopD yscK* (▽) mutants in chemically defined medium with 100 mM Na⁺ and 25 mM L-glutamic acid (pH 7.5) in the presence (a) or absence (b) of added 4.0 mM Ca^{2+} .

Na^+ leakage. Accordingly, our findings support the general hypothesis that operation of the T3SS causes toxicity that can be relieved by omitting Na^+ from the medium and that this phenomenon is manifested as the relief by Ca^{2+} of growth restriction historically known as the LCR. At the time of writing, there is no evidence, to our knowledge, for an involvement of Na^+ in the workings of the T3SS (Wilhelm *et al.*, 2004), and future study will be required to determine how the T3SS facilitates uptake of this cation.

The present study identified YscV as a suppressor of the truly temperature-sensitive mutation in *yopD*. This finding indicates that the temperature-sensitivity of a YopD⁻ mutant is linked to the operation of the core translocon and specifically to the YscV function. Because *yscK* and apparently also *yscR* (unpublished data), *yscC* and *yscD* mutations failed to suppress the *yopD* growth phenotype, a YscV function distinct from secretion per se is implicated in the temperature-sensitivity of the *yopD* strain. YopD is a major negative controller of T3SS function, from expression to the ordered secretion of T3SS substrates (Anderson *et al.*, 2002; Francis *et al.*, 2001; Williams & Straley, 1998; Wulff-Strobel *et al.*, 2002), and is associated with the bacterial membrane fraction, whether Ca^{2+} is present or not (Williams & Straley, 1998). Perhaps a YopD-associated T3SS negative regulatory module interacts with YscV to synchronize energy flow from the YscN ATPase with substrate availability. In this context, loss of YopD might cause uncoupling of YscN from the T3SS, resulting in a futile energy-draining ATPase cycle.

The work described above provides important insights into the assimilation of Na^+ by Ca^{2+} -deprived wild-type cells of *Y. pestis* but does not account for bacteriostasis or the attendant exit of L-aspartic acid. It is significant, however, that Na^+ serves as a porter for L-glutamate and L-aspartate transport (Deguchi *et al.*, 1989; Kalman *et al.*, 1991; MacDonald *et al.*, 1977; Schellenberg & Furlong, 1977); thus, the possibility exists that the export of aspartic acid (caused by deficiency of AspA in *Y. pestis*) is favoured by Na^+ internalized during upregulation of the T3SS. In conclusion, the present findings emphasize that Lcr⁺ yersiniae do not possess a nutritional requirement for Ca^{2+} per se but rather need this cation to alleviate sensitivity to Na^+ . This relationship remained undiscovered until recently because Na^+ is a favoured cationic equivalent used to prepare synthetic media, a common component of chelators used to eliminate Ca^{2+} (e.g. sodium oxalate and sodium EDTA), and a ubiquitous ingredient of media prepared from natural sources. This form of Na^+ toxicity may not occur *in vivo* because significant levels of the cation only occur within extracellular fluids that also contain sufficient Ca^{2+} (Kugelmass, 1959) to downregulate expression of T3SS virulence effectors (Brubaker & Surgalla, 1964; Portnoy *et al.*, 1981; Straley & Brubaker, 1981). Mammalian cytoplasm, whether present in favoured niches such as intact host cells or closed necrotic lesions within visceral organs, is deficient in both Ca^{2+} and Na^+ (Kugelmass, 1959) and thus suitable for sustained growth with concomitant expression of Yops and LcrV.

Fig. 9. Immunoblots (YopH, YopD and LcrV) and silver-stained preparations following SDS-PAGE of whole cultures of Lcr⁻ (lane 1), Lcr⁺ (lane 2), *yopD* (lane 3), *yscK* (lane 4), *yscV* (lane 5), *yscK yopD* (lane 6) and *yscV yopD* (lane 7) yersiniae after 7 h incubation at 37 °C in chemically defined medium with 100 mM Na⁺ and 25 mM L-glutamic acid (pH 7.5) either containing (a) or lacking (b) 4.0 mM Ca²⁺; also shown in both panels are Ca²⁺-deficient control cultures of Lcr⁺ cells following growth for 9 h in the same medium without added Ca²⁺ (lane 8).

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