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.

INTRODUCTION

The medically significant *yersinia* 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; Kuperberg & 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–YaeA (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 *yersinia* 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...
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$^+$) yersinia cells bind more carrier-free $^{45}$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$^+$ yersinia 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 yersinia, 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 yapN but not yapD 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 (Besseley 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 (Berber & Brubaker, 1981). Retention of pPCP was monitored by expression of pesticin on blood agar base (Difco) using Y. pseudotuberculosis strain PBI 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 Ysc313-A (5' CGGATTACGCAAAATTGTGACACGGATTTGGGTACCCATCACTTCT 3') and YskKapn-B (5' GACAGTTTTGGAACATTGTTGACACGAATTCAGC 3') were used to amplify a 776 bp region upstream of yscK and introduce unique EcoRI and KpnI restriction sites. Primers YskKapn-A (5' TGGTTCGAAACCGAAATGAGTGTTGATGCAGCCAACTTTATGTCACAACTTCT 3'), complementary to YskKapn-B, and YscL+63-B (5' CGGATCCCATTTAGTTGATGATGAACTTACCTGACTACTGACTACTGACTAC 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 pLDD5. Because yscL overlaps yscK, the design of the YskKapn 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(p). Deletion vector pAW211 was used to delete yscK from the LCR plasmid (pCD1) by allelic exchange, as described previously (Metcalfe 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 arabino- inducible promoter in pYscKHA18. To make this plasmid, the 630 bp yscK gene was amplified with primers YskKISD-A (5' GCCAGTGGACGCGAAGAACATATGGAAATTATTTATCTC 3') and YskK630HA-B (5' GATCCTAGATATCAATCTGGAACACTCATGATGATGAAACTTACCTGACTACTGACTAC 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 Xbal restriction site. The resultant PCR product was purified, digested with SacI and Xbal, and ligated into pBAD18-Km' (Guzman et al., 1995) that had been digested with SacI and Xbal. The resulting pYscKHA18 plasmid was electroporated into ΔyscK Y. pestis KIM-3002.12.

To make the complementation test, the parent YscK $^+$ Y. pestis KIM8-3002.12 and Y. pestis KIM8-3002.2.2 were grown for approximately seven generations at 26 °C in the defined
medium TMH containing 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 (Wulf-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 pTrcM2.2 (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 ΔyscK bacteria failed to show growth restriction, in contrast to the parental 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 gyratory 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 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

Table 1. Bacterial strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description*†</th>
<th>Source or reference</th>
</tr>
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<tr>
<td><strong>Y. pestis strains</strong></td>
<td></td>
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</tr>
<tr>
<td>KIM5</td>
<td>pCD1, pPCP1, pMT1; Lcr+; Pgm+ wild-type parental strain; also called D27</td>
<td>R. R. Brubaker</td>
</tr>
<tr>
<td>KIM6</td>
<td>Lcr- (pCD1-), pPCP1, pMT1; also called D28</td>
<td>R. R. Brubaker</td>
</tr>
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<td>KIMB-3002</td>
<td>Sm8; pCD1 (Lcr+, Pla- (pPCP1-), pMT1</td>
<td>Nilles et al. (1998); unpublished data</td>
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<td>KIMB-3002.7</td>
<td>Sm8; pCD1 (ΔlcrG3 [6–86], Pla- (pPCP1-), pMT1</td>
<td>Fields et al. (1999)</td>
</tr>
<tr>
<td>KIM-3000.6</td>
<td>Sm8; pCD1 (ΔyopN [48–197]), pPCP1, pMT1</td>
<td>Plano &amp; Straley (1995)</td>
</tr>
<tr>
<td>KIM-3-3401</td>
<td>Sm8; pCD1 (krH-::cat, yopI::Mudl1734 [Km2 Lac+ LcrH- YopB- YopD- YopJ]), pPCP1, pMT1</td>
<td>Price &amp; Straley (1989)</td>
</tr>
<tr>
<td>KIM-3-3241.2</td>
<td>Sm8 Km2 Lac+; pCD1 (ΔlcrV [18–215], yopI::Mudl1734), pPCP1, pMT1</td>
<td>Price et al. (1991)</td>
</tr>
<tr>
<td>KIMB-3002.2</td>
<td>Sm8; pCD1 (ΔyopD [1–305], Pla- (pPCP1-), pMT1</td>
<td>Williams &amp; Straley (1998)</td>
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<td>KIM-3000.1.2.1</td>
<td>Sm8; pCD1 (ΔyscV [192–343] ΔyopD [1–305]), pPCP1, pMT1</td>
<td>Williams &amp; Straley (1998)</td>
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<tr>
<td>KIMB-3002.12</td>
<td>Sm8; pCD1 (ΔyscK [4–190], Pla- (pPCP1-), pMT1</td>
<td>This study</td>
</tr>
<tr>
<td>KIMB-3002.2.2</td>
<td>Sm8; pCD1 (ΔyopD [1–305], ΔyscK [4–190] Pla- (pPCP1-), pMT1</td>
<td>This study</td>
</tr>
<tr>
<td><strong>E. coli strain</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SY327 (λpir)</td>
<td>Δ(lac-pro) argE(Am) rif nala recA56 λpir</td>
<td>Miller &amp; Mekalanos (1988)</td>
</tr>
</tbody>
</table>

*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).
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 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.
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 component 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. 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.
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 Ca\(^{2+}\). 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

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**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).
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. 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 (Cal1) (open arrows) varied in amount among the strains and correlated with their net growth following shift from 26 to 37 °C.
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 Ca²⁺; also shown in both panels are Ca²⁺-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 Ca²⁺; also shown in both panels are Ca²⁺-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).
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 Ca²⁺-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. 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 Ca²⁺; also shown in both panels are Ca²⁺-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).
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 (Wilharm 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; Wulf-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\(^{2+}\) (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. 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\(^{2+}\).](image-url)
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