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Cell Reports
Report

The Central Metabolism Regulator EIIA^{Glc} Switches *Salmonella* from Growth Arrest to Acute Virulence through Activation of Virulence Factor Secretion

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http://dx.doi.org/10.1016/j.celrep.2014.04.022

SUMMARY

The ability of *Salmonella* to cause disease depends on metabolic activities and virulence factors. Here, we show that a key metabolic protein, EIIA^{Glc}, is absolutely essential for acute infection, but not for *Salmonella* survival, in a mouse typhoid fever model. Surprisingly, phosphorylation-dependent EIIA^{Glc} functions, including carbohydrate transport and activation of adenylate cyclase for global regulation, do not explain this virulence phenotype. Instead, biochemical studies, in vitro secretion and translocation assays, and in vivo genetic epistasis experiments suggest that EIIA^{Glc} binds to the type three secretion system 2 (TTSS-2) involved in systemic virulence, stabilizes its cytoplasmic part including the crucial TTSS-2 ATPase, and activates virulence factor secretion. This unexpected role of EIIA^{Glc} reveals a striking direct link between central *Salmonella* metabolism and a crucial virulence mechanism.

INTRODUCTION

Salmonella enterica causes diarrhea but also more life-threatening systemic diseases such as typhoid fever in humans and animals (Coburn et al., 2007). *Salmonella* growth and disease progression depends on *Salmonella* metabolic activities and virulence factors (Haraga et al., 2008; Kuhle and Hensel, 2004; Steeb et al., 2013), and both types of activities are likely coordinated during infection (Görke and Stülke, 2008; Poncet et al., 2009).

Phosphoenolpyruvate:carbohydrate phosphotransfer transport systems (PTSs) may participate in this coordination because they mediate nutrient uptake, control global metabolism, and modulate virulence in diverse pathogens (Deutscher et al., 2006; Poncet et al., 2009). PTSs catalyze sugar uptake through sugar-specific permeases called enzymes II with cytoplasmic domains EIIA and EIIB and membrane-crossing domain EIIC (and sometimes EIID). During transport, the sugar is phosphorylated by phosphoenolpyruvate (PEP) through intermediary phosphate acceptors including general enzyme I and HPr. The phosphorylation status of PTS components depends on availability of sugar substrates and glycolysis/gluconeogenesis fluxes that determine the PEP/pyruvate ratio. PTS phosphorylation thus reflects the general metabolic state, and this is exploited by many bacteria as sensory input to regulate metabolism, respiration, motility, and virulence (Deutscher et al., 2006).

As an example, the PTS component EIIA^{Glc} (encoded by the carbohydrate repression resistance gene, *crr*) mediates uptake of glucose, N-acetyl-muramic acid, and yet unidentified sugars. In absence of these substrates, phosphorylated EIIA^{Glc} (EIIA^{Glc}-P) accumulates and activates adenylate cyclase (CyaA), resulting in increased cyclic AMP (cAMP) levels. cAMP activates the cAMP receptor protein (CRP; also called catabolite gene activator protein), a transcription factor that modulates expression of several hundred genes (Deutscher et al., 2006). In addition to CyaA, EIIA^{Glc} directly modulates the activity of several other metabolic proteins including glycerol kinase (Hurley et al., 1993), the fermentation/respiration switch protein (FrsA) (Koo et al., 2004), PtsN (Rabus et al., 1999), and MalK (Chen et al., 2013).

In this study, we discovered a major role of EIIA^{Glc} in *Salmonella* virulence in a mouse infection model that closely mimics human typhoid fever (Santos et al., 2001).

RESULTS

EIIA^{Glc} Is Essential for Systemic *Salmonella* Virulence

Salmonella crr lacking the gene encoding EIIA^{Glc}, has a moderate growth defect in cell culture infections (Bowden et al., 2009). To determine the in vivo relevance of EIIA^{Glc}, we infected mice with a mixture of wild-type *Salmonella enterica* serovar Typhimurium SL1344 and a *crr* deletion mutant. Wild-type outcompeted the *crr* mutant 250-fold within 3 days, indicating very strong attenuation, and *crr* complementation in *cis* or *trans* restored full virulence (Figure 1A). The *crr* mutant failed to grow in spleen but survived for at least 28 days (Figures 1B and 1C). In contrast, *Salmonella crr* grew normally in vitro (Figure S1A). These data establish that EIIA^{Glc} has a crucial role in *Salmonella* virulence.

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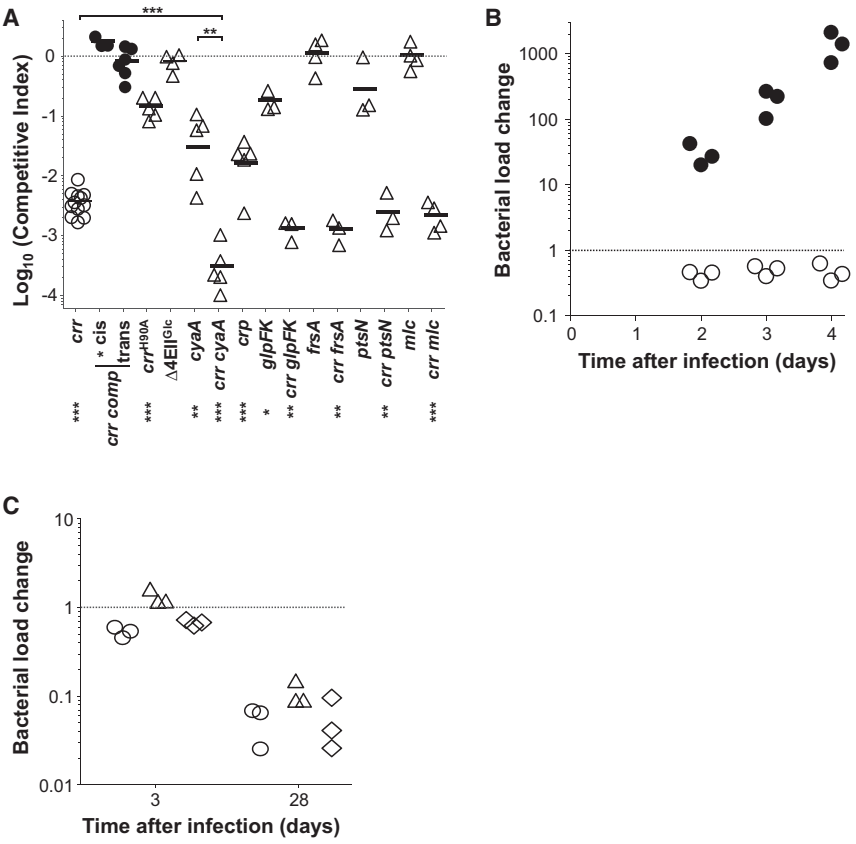

Figure 1. Virulence of *Salmonella crr* and Related Mutants

(A) Competitive indices of *Salmonella* mutants versus wild-type at day 3 postinfection in mouse spleen. A $\log_{10}$(competitive index [CI]) value of 0 (equivalent to a CI of 1) represents full virulence. Data for individual mice and geometric means are shown. Statistical significance of differences against wild-type or between mutant pairs was tested using t test on log-transformed data (***$p < 0.001$; **$p < 0.01$; *$p < 0.05$).

(B) Time course for *Salmonella crr* (open circles) and wild-type *Salmonella* (filled circles). Spleen loads of individual mice relative to the infection doses are shown.

(C) Spleen loads for *Salmonella crr* (open circles), *ssaGH* (triangles), and *crr ssaGH* (diamonds) relative to the infection doses.

See also Figure S1.

Known EIIA^{Glc} Functions Do Not Explain Its Role in Virulence

EIIA^{Glc} mediates PTS sugar uptake, but this function was dispensable for *Salmonella* virulence because a *ptsG murP nagE SL1344_2742* (Δ4EII^{Glc}) mutant lacking all EIIA^{Glc}-dependent EIIBC systems as well as NagE (a glucose-type EIICBA that interacts with EIIA^{Glc}; Vogler and Lengeler, 1988) retained wild-type virulence (Figure 1A). This was confirmed by the high virulence of *Salmonella* carrying a *crr*^{H90A} allele encoding an EIIA^{Glc} variant that lacks the crucial phosphorylation site histidine 90 required for sugar transport (Dörschug et al., 1984; Figure 1A).

EIIA^{Glc}-P modulates *Salmonella* gene expression through activation of CyaA and CRP (both of which are essential for *Salmonella* virulence; Curtiss and Kelly, 1987). However, the high virulence of *Salmonella crr*^{H90A} (Figure 1A) suggested that this and other phosphorylation-dependent EIIA^{Glc} functions were largely dispensable. This was confirmed by the additive defects of *cyaA* and *crr* mutations in a *cyaA crr* double mutant (Figure 1A), indicating EIIA^{Glc} functions other than CyaA activation. cAMP and cAMP/CRP are crucial for virulence but can apparently be supported by basal CyaA activities in absence of EIIA^{Glc}-P (Nelson et al., 1982; You et al., 2013). Indeed, *Salmonella crr* and *Salmonella crr*^{H90A} carrying a CRP-dependent promoter-*gfp* fusion had substantial GFP fluorescence in vitro and in vivo, confirming cAMP/CRP activity in absence of EIIA^{Glc}-P (Figures S1C and S1D). Similarly, *Salmonella* utilization of several relevant nutrients (Steeb et al., 2013) was CRP dependent but largely EIIA^{Glc} independent (Figure S1E). Finally, proteome comparisons under conditions mimicking in vivo environments revealed 19 EIIA^{Glc}-regulated proteins out of 1,254 detected proteins (Table S1), but none of them was likely to mediate the *crr* virulence phenotype (Table S1). These data did not support a critical virulence role of EIIA^{Glc} in gene regulation in contrast to other PTS components (*Salmonella* EIIBC^{Glc}/type three secretion system [TTSS]-1 [Lim et al., 2007]; *Salmonella* EIIA^{Ntr}/SsrB [Choi et al., 2010]; *Brucella melitensis* HPr and enzyme I/type IV secretion [Dozot et al., 2010]).

EIIA^{Glc} modulates the activity of additional proteins. Analysis of corresponding *Salmonella* single and double mutants suggested that none of these interactions could individually explain the strong *crr* virulence defect (Figure 1A). This included the EIIA^{Glc} paralog EIIA^{Ntr}, which was largely dispensable for *Salmonella* systemic virulence, in contrast to oral infections (Choi et al., 2010). However, these data did not rule out that simultaneous EIIA^{Glc} interactions with several partners might together support *Salmonella* virulence.

EIIA^{Glc} Interacts with the Type III Secretion System Encoded on SPI-2

EIIA^{Glc} might interact with yet unknown proteins. To explore this issue, we performed coimmunoprecipitation/mass spectrometry (coIP/MS) with *Salmonella* expressing 3×FLAG-tagged EIIA^{Glc}. Comparison with data for a control strain without FLAG-tagged protein revealed specific enrichment of known interaction partners such as EIICB^{Glc} (encoded by *ptsG*; Buhr et al., 1994) and FrsA (Koo et al., 2004), as well as many potential new interaction partners (Table S2), similar to findings for *Vibrio cholerae* EIIA^{Glc} (Pickering et al., 2012). Using a cutoff of five identified peptides, 79 proteins were exclusively found in EIIA^{Glc}-FLAG coIPs, whereas just three were exclusively found in the controls. Among the identified 79 specific candidates, there were several subunits

of the type III secretion system encoded on the *Salmonella* pathogenicity island 2 (TTSS-2), which is essential for *Salmonella* virulence (Shea et al., 1996; Figure 2B; Table S2).

It is important to note that we crosslinked complexes using mild formaldehyde treatment to stabilize them during purification. The identified TTSS-2 components could thus represent direct interaction partners of EIIA^{Glc} or crosslinked proteins from the same complexes with no direct interaction. These data have additional caveats: (1) Our controls helped to exclude false-positive proteins that bind to the anti-FLAG beads used for immunoprecipitation (IP), but we could not rule out that some proteins might bind to the FLAG tag on EIIA^{Glc}. (2) We failed to detect known interaction partners GlpK, HPr, CyaA, MelB, and MalK, indicating important false negatives. CyaA, MalK, and MelB were poorly expressed under our experimental conditions. GlpK and HPr were found in similar amounts in coIPs of *Salmonella* expressing EIIA^{Glc}-FLAG and controls indicating nonspecific binding. The coIP data thus provided only a first hint about potential interactions with TTSS-2.

Salmonella crr assembled apparently normal TTSS-2 SsaCDJPRVU core complexes that could be captured with 3× FLAG-tagged SsaD (Figures 2A and 2B; Table S3). However, several more peripheral subunits were underrepresented compared to TTSS-2 from wild-type. This included SsaK, SsaQ, SsaO, SL1344_1344, and the ATPase SsaN, which is essential for secretion (Cooper et al., 2010; Yoshida et al., 2014). All these subunits probably localize to the cytoplasmic part of TTSS-2 (Rey et al., 2005; Yoshida et al., 2014), where they could interact with EIIA^{Glc} (Wang et al., 2000).

SsaK, SsaN, and SsaO might interact directly based on the EIIA^{Glc}-FLAG coIP data (Figure 2B), whereas SL1344_1344 and SsaO were not found to interact with EIIA^{Glc} but might bind to SsaK, SsaN, or SsaO, or their TTSS-2 association may depend on EIIA^{Glc}-dependent conformational TTSS-2 states. Several other components that were pulled down together with EIIA^{Glc} were assembled in TTSS-2, regardless of EIIA^{Glc}. This could reflect pull-down of large crosslinked TTSS-2 complexes that included components with no direct interactions to EIIA^{Glc}. Finally, the secreted translocon component SseB was also diminished in *Salmonella crr* TTSS-2 complexes, and this might reflect compromised secretion activity in the absence of essential cytoplasmic subunits.

Underrepresentation of some TTSS-2 subunits was probably not the result of poor expression. SseB and SsaQ were actually detected at similar levels in cell lysates of *crr* and wild-type (Table S1). SsaK and SL1344_1344 were not detected in cell lysates presumably because of high sample complexity, but these subunits are known to be coexpressed from the same operon (Walthers et al., 2007) as normally abundant SsaH (Table S1) and normally assembling SsaJ (Table S3). Similarly, SsaN and SsaO were not detected in lysates, but they are coexpressed with normally abundant SsaQ and SsaU (Table S1) and normally assembled SsaPRUV (Table S3; Walthers et al., 2007).

To further explore EIIA^{Glc}/TTSS-2 interactions, we used a bacterial two-hybrid system (Karimova et al., 1998) in *Salmonella* with libraries containing fragments of all known TTSS-2-associated genes. We did not find interactions between EIIA^{Glc} and structural TTSS-2 proteins using this approach. This could indicate no direct interaction or non-native conformations of TTSS-2 components and their fragments in fusion proteins that were not assembled in intact TTSS-2. On the other hand, we detected EIIA^{Glc} interactions to N-terminal fragments of the TTSS-2 effectors PipB and PipB2 (Figure 2C). Interactions were similar for full-length EIIA^{Glc} and EIIA^{Glc} ΔN16 lacking the first 16 amino acids required for membrane association (Wang et al., 2000; Figure S2A). This and other data suggested that interactions with PipB and PipB2 may play a subordinate part in the crucial virulence function of EIIA^{Glc} (see below). We therefore did not independently verify these interactions.

Together, these data showed that EIIA^{Glc} had limited impact on TTSS-2 expression or assembly of the core secretion apparatus but stabilized the crucial cytosolic part of TTSS-2 and might interact with some effectors.

EIIA^{Glc} Is Essential for TTSS-2 Effector Secretion

To determine the impact of EIIA^{Glc} on TTSS-2 function, we analyzed effector secretion and translocation. Wild-type *Salmonella*, a *crr*^{H90A} mutant (no EIIA^{Glc} phosphorylation), and a *crr*^{H90D} mutant (potentially mimicking EIIA^{Glc}-P) all secreted similar amounts of effectors into axenic culture supernatants (Figure 3A). Similarly, available carbon sources strongly affected EIIA^{Glc} phosphorylation status in wild-type *Salmonella*, as expected (Figure S2B), but this had no detectable impact on TTSS-2 secretion (Figures 3A and S2B). However, secretion was completely abolished in Δ*crr* (Figure 3A) but restored to elevated levels by complementation with multicopy *crr* (Figure S2C). EIIA^{Glc} interactions with effectors PipB and PipB2 (see above) were not required for TTSS-2 secretion as demonstrated by normal SteC secretion in *Salmonella pipB*, *pipB2*, and *pipB pipB2* mutants (Figure S2D).

Salmonella wild-type and *crr*^{H90A}, but not Δ*crr*, translocated M45-tagged PipB2 (Halici et al., 2008) into the cytoplasm of infected macrophages (Figure 3B). EIIA^{Glc} D38A or EIIA^{Glc} D94A with surface alterations close to the phosphorylation site at histidine 90 (Worthylake et al., 1991) enabled normal PipB2 translocation. In contrast, translocation was abolished in *Salmonella* expressing EIIA^{Glc} D38A F41A K69A D94A or EIIA^{Glc} ΔN16 (Figure 3C), despite substantial EIIA^{Glc} protein levels in both strains (Figure S2E). These translocation effects of EIIA^{Glc} mutations differed from effects on a cAMP/CRP-dependent promoter fusion (Figure 3C), indicating distinct EIIA^{Glc} interaction surfaces for TTSS-2 and CyaA.

These data show that EIIA^{Glc} is required for secretion through TTSS-2, whereas secretion of TTSS-1 effectors and flagellin is independent of EIIA^{Glc} (Figure S2F). TTSS-2 can be activated by both nonphosphorylated EIIA^{Glc} H90A and EIIA^{Glc} H90D carrying a negative charge at the normal phosphorylation site, but if EIIA^{Glc}-P would also activate remains unclear. To address this issue, we would need assay conditions where *Salmonella* contains exclusively EIIA^{Glc}-P, but our attempts in this direction using succinate medium (Hogema et al., 1998) were unsuccessful (Figure S2B).

Activation of TTSS-2 Is the Major Virulence Function of EIIA^{Glc} in Systemic Salmonellosis

Loss of TTSS-2 activity could explain the strong attenuation of *Salmonella crr*. To test this hypothesis, we analyzed genetic

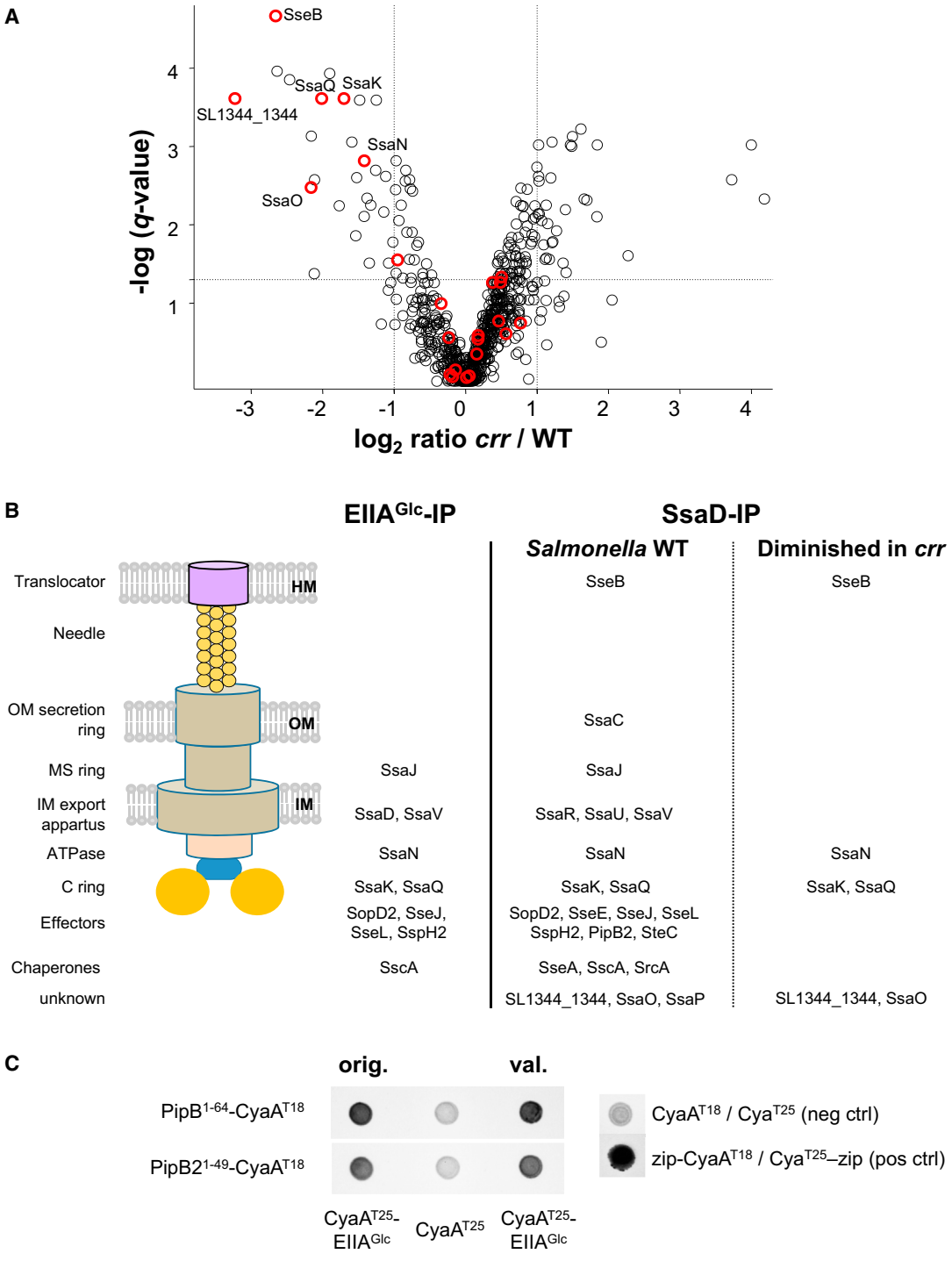

Figure 2. EIIA^{Glc} Interaction with *Salmonella* Type Three Secretion System 2

(A) Proteins recovered by SsaD coIP in *Salmonella* wild-type- and *crr*. TTSS-2-associated proteins are labeled with red circles. Proteins with a >2-fold change and q value < 0.05 are considered significant (upper left and right areas).

(B) Schematic representation of TTSS-2 and likely positions of proteins detected by coIP with EIIA^{Glc}-3×FLAG or TTSS-2 subunit SsaD-3×FLAG in *Salmonella* wild-type (WT) and *crr*. See also Tables S1, S2, and S3. IM, inner membrane.

(C) Interaction of EIIA^{Glc} with TTSS-2 effector proteins PipB and PipB2 as detected by bacterial two-hybrid screening. Plasmids were isolated from strains with positive staining ("orig.") and transformed into a strain without CyaA^{T25}-EIIA^{Glc} (CyaA^{T25}, control for unspecific activity) or retransformed into another strain expressing CyaA^{T25}-EIIA^{Glc} ("val.") to demonstrate that CyaA activity was due to protein interactions instead of secondary mutations in the initial clone. Controls ("neg ctrl," no interaction; "pos ctrl," known interaction) are also shown.

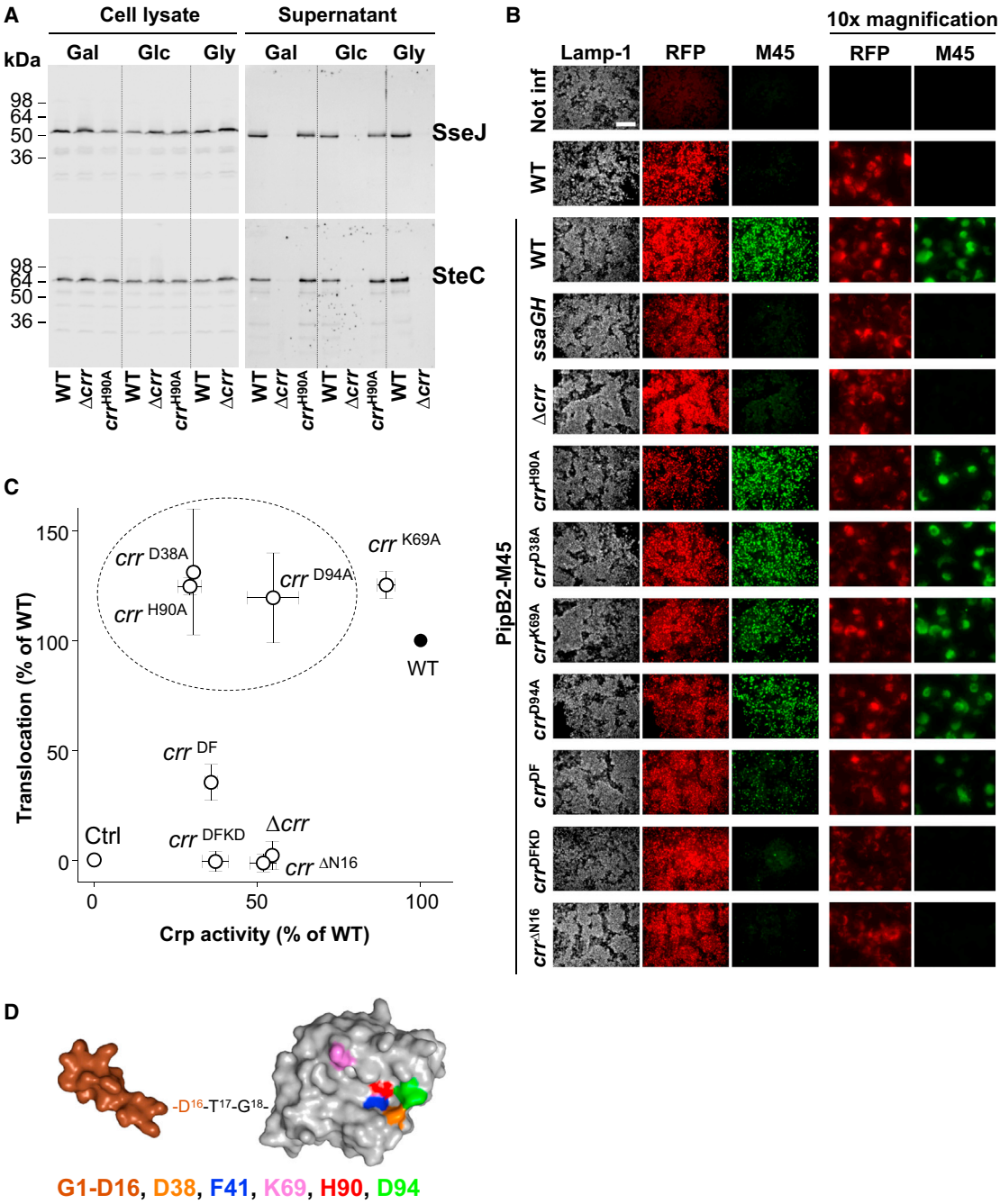

Figure 3. EIIA^{Glc} Activation of *Salmonella* Type Three Secretion System 2

(A) Immunoblots for 3×FLAG-tagged TTSS-2 effectors SseJ and SteC in *Salmonella* WT, *crr*, and *crr*^{H90A} in minimal media containing various carbon sources (Gal, galactose; Glc, glucose; Gly, glycerol). *Salmonella crr*^{H90A} is omitted for glycerol because of its poor growth on this carbon source.

(B) Translocation of M45-tagged PipB2 by *Salmonella* strains expressing the red fluorescent protein mCherry (RFP) and various variants of EIIA^{Glc} (DF, D38A F41A; DFKD, D38A F41A K90A D94A; ΔN16, EIIA^{Glc} lacking the N-terminal amino acids 1–16) in RAW macrophage-like cells. Immunostaining of LAMP-1 is used as a host cell marker. Representative images from three similar independent experiments are shown. The scale bar represents 200 μm.

(C) Translocation efficiencies and cAMP/CRP-dependent promoter activities in *Salmonella* strains expressing various EIIA^{Glc} variants. The dashed ellipse highlights EAII^{Glc} variants with diminished CRP activities but unimpaired translocation. Mean values and SEMs from three independent experiments each are shown.

(D) Position of mutated residues in EIIA^{Glc}. Structures of the major C-terminal fragment and a short N-terminal peptide have been determined separately, but their connection is unclear.

See also Figure S2.

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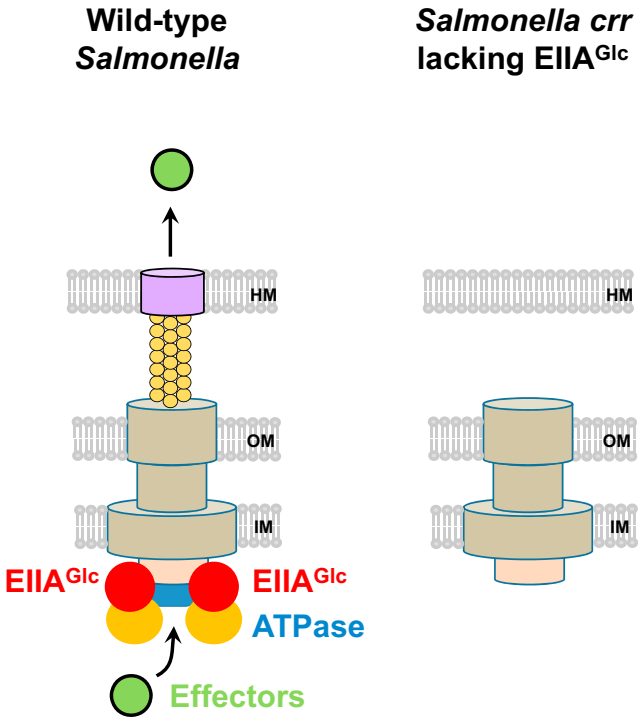

Figure 4. Model of EIIA^{Glc} Activation of *Salmonella* Type Three Secretion System 2

EIIA^{Glc} stabilizes the cytosolic part of the secretion apparatus and activates secretion of effectors.

epistasis between *crr* and TTSS-2 defects. A *crr* mutant, a *ssaGH* mutant lacking crucial structural components of TTSS-2, and a *crr ssaGH* double mutant all had similarly strong virulence defects but survived in spleen for 28 days (Figure 1C). The lack of additive attenuating effects of *ssaGH* was not due to already completely abrogated virulence of *Salmonella crr*, because a *cyaA* mutant in the same *crr* background actually did show exacerbated attenuation (Figure 1A). Similarly, the individually strongly attenuating *crr* mutation had a very minor additive effect in *ssaGH*, in contrast to other mutations such as *phoP* that show strong additive effects in TTSS-2 mutants such as *ssrB* (Beuzón et al., 2001). In absence of a functional TTSS-2, EIIA^{Glc} is thus largely irrelevant, whereas in absence of EIIA^{Glc}, TTSS-2 has no impact on *Salmonella* virulence. These findings strongly suggest that EIIA^{Glc} and TTSS-2 virulence functions are mutually dependent on each other in agreement with our findings of EIIA^{Glc}-dependent TTSS-2 secretion/translocation. Major other EIIA^{Glc} functions apart from TTSS-2 activation are unlikely based on similar attenuation of *ssaGH* and *ssaGH crr*.

In conclusion, EIIA^{Glc}-dependent TTSS-2 activation was necessary and sufficient to explain the crucial role of EIIA^{Glc} in *Salmonella* virulence.

DISCUSSION

This study revealed an unexpected crucial role of the sugar transport protein and metabolic regulator EIIA^{Glc} in *Salmonella* virulence. Specifically, EIIA^{Glc} stabilized the crucial cytosolic part of TTSS-2 and activated TTSS-2 virulence factor secretion (Figure 4). Further studies are required to unravel the detailed mechanisms of activation. This is currently hampered by lacking protocols for purifying functional TTSS-2.

EIIA^{Glc} might also interact with effectors PipB and PipB2, but this was at most a subordinate function because TTSS-2 secretion was unimpaired in *Salmonella pipB pipB2* (Figure S2D). Moreover, EAII^{Glc} ΔN16 interacted equally well with PipB and PipB2 (Figure S2A), although this EIIA^{Glc} variant did not support TTSS-2 translocation (Figures 3B and 3C). EIIA^{Glc} interactions with PipB and PipB2 are thus dispensable and insufficient for TTSS-2 activation, consistent with weak *pipB* and *pipB2* virulence phenotypes (Knodler et al., 2003; Wood et al., 1998).

EIIA^{Glc} is an abundant protein during infection (Steeb et al., 2013) and might thus be readily available as a scaffold for TTSS-2 structural stabilization. In addition, *Salmonella* might use EIIA^{Glc} as a metabolic sensor to adjust TTSS-2 activity and, thereby, virulence/persistence, similarly to TTSS activation by pH in *Salmonella* (McGourty et al., 2012) or oxygen tension in *Shigella* (Marteyn et al., 2010). Purified functional TTSS-2 complexes and pure EIIA^{Glc} or EIIA^{Glc}-P could help to test these and alternative hypotheses in future studies.

EIIA^{Glc} represents a potential target for *Salmonella* control. This had not previously been recognized due to weak phenotypes in axenic and cell culture models, but this infection-specific role could actually be beneficial as inhibition might have little impact on normal microbiota, whereas abolishing *Salmonella* virulence.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Antibodies

Strains derived from *Salmonella enterica* serovar Typhimurium SL1344 (Hoiseth and Stocker, 1981), plasmids, and antibodies used in this study are listed in Table S4. Mutants were constructed using lambda red recombinase (Datsenko and Wanner, 2000) as described in the Supplemental Experimental Procedures.

Mouse Infections

All animal experiments were approved by Kantonales Veterinäramt Basel-Stadt (license 2239) and performed according to local guidelines (Tierschutz-Verordnung, Basel-Stadt) and the Swiss animal protection law (Tierschutz-Gesetz). BALB/c mice were infected intravenously with *Salmonella* as described in the Supplemental Experimental Procedures.

Protein Secretion Assay

TTSS-2 secretion was assayed by precipitation of culture supernatants 90 min after a pH shift from 5.0 to 7.0 (Yu et al., 2010), followed by western blotting as described in the Supplemental Experimental Procedures.

Coimmunoprecipitation and Mass Spectrometry

Salmonella wild-type and *crr-FLAG* were cultured in 2-(N-morpholino)ethanesulfonic acid (MES)-buffered medium (pH 5.0) and fixed with formaldehyde. *Salmonella* were lysed and incubated with anti-FLAG M2 agarose beads. After washing, bound proteins were eluted and processed for proteomics as described in Supplemental Experimental Procedures.

Proteome Comparison

Salmonella wild-type and *crr* were cultured in MES-buffered medium (pH 5.0) containing 0.4% of glycerol, processed for proteomics, and analyzed on

nano-liquid chromatography-tandem mass spectrometry as described in Supplemental Experimental Procedures.

Bacterial Two-Hybrid Screening

Genomic fragments encoding TTSS-2-associated genes were cloned in pUT18 (Karimova et al., 1998). *crr* was fused in frame to the C terminus of T25 (Karimova et al., 1998), yielding pEIIA^{Glc}-T25. pKT25-zip and pUT18C-zip were used as positive controls. Interactions were screened in *Salmonella crr cyaA* to minimize background. Our analysis was restricted to qualitative staining patterns on plates because standard β-galactosidase assays could not be used in *Salmonella* that lacks this enzyme, and attempts to develop alternative quantitative assays failed.

Macrophage Cell Culture Infections

RAW264.7 macrophages were infected and analyzed for TTSS-2 effector translocation using immunofluorescence as described in Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, two figures, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.04.022.

AUTHOR CONTRIBUTIONS

A.M. designed and performed experiments, analyzed data, and wrote the paper; T.G. performed all mass spectrometry experiments and analyzed data; and D.B. designed experiments, analyzed data, and wrote the paper.

ACKNOWLEDGMENTS

We thank N. Figueroa-Bossi for providing pSUB11, K. Bettenbrock for pSC26-*scrY*p, M. Hensel for p3181, and K. Jahreis for a polyclonal antibody to EIIA^{Glc}. We thank B. Claudi, N. Burton, M. Rosas Ballina, and C. Kasper for their help with experimental methods. This project was in part funded by Swiss National Foundation (31003A-121834).

Received: August 14, 2013
Revised: March 7, 2014
Accepted: April 14, 2014
Published: May 15, 2014

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Please cite this article in press as: Mazé et al., The Central Metabolism Regulator EIIA^{Glc} Switches *Salmonella* from Growth Arrest to Acute Virulence through Activation of Virulence Factor Secretion, Cell Reports (2014), http://dx.doi.org/10.1016/j.celrep.2014.04.022

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