Streptococcus pyogenes upregulates arginine catabolism to exert its pathogenesis on the skin surface

Highlights

- *S. pyogenes* uses arginine catabolism under low-glucose conditions
- Arginine catabolism contributes to its viability and virulence on skin surface
- Arginine catabolism is suppressed under high-glucose conditions in blood
- *S. pyogenes* acquires arginine from the stratum-corneum-derived filaggrin

In brief

Hirose et al. show that *S. pyogenes* upregulates arginine catabolism to exert its pathogenesis under low-glucose conditions. *S. pyogenes* changes global gene expression, including upregulation of virulence genes, by catabolizing arginine. *S. pyogenes* acquires arginine from stratum-corneum-derived filaggrin to adapt to glucose starvation on the skin.
Streptococcus pyogenes upregulates arginine catabolism to exert its pathogenesis on the skin surface

Yujiro Hirose,1,2,11 Masaya Yamaguchi,1 Tomoko Sumitomo,1 Masanobu Nakata,3 Tomoki Hanada,1 Daisuke Okuzaki,4 Daisuke Motooka,5 Yasushi Mori,1 Hiroshi Kawasaki,6,7,8 Alison Coady,2 Satoshi Uchiyama,2 Masanobu Hiraoka,2,9 Raymond H. Zurich,2 Masayuki Amagai,6,8 Victor Nizet,2,10 and Shigetada Kawabata1,*
1Department of Oral and Molecular Microbiology, Osaka University Graduate School of Dentistry, Suita, Osaka 565-0871, Japan
2Department of Pediatrics, University of California at San Diego School of Medicine, La Jolla, CA 92093, USA
3Department of Oral Microbiology, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima 890-8544, Japan
4Genome Information Research Center, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka 565-0871, Japan
5Department of Infection Metagenomics, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka 565-0871, Japan
6Department of Dermatology, Keio University School of Medicine, Tokyo 160-8582, Japan
7Immunology Data Integration Unit, RIKEN Medical Sciences Innovation Hub Program, Yokohama 230-0045, Japan
8Laboratory for Skin Homeostasis, RIKEN Center for Integrative Medical Sciences, Yokohama 230-0045, Japan
9Department of Otorhinolaryngology-Head and Neck Surgery, Wakayama Medical University, Wakayama, Wakayama 641-8509, Japan
10Skaggs School of Pharmaceutical Sciences, University of California at San Diego, La Jolla, CA 92093, USA
11Lead contact
*Correspondence: yujirohirose@dent.osaka-u.ac.jp (Y.H.), kawabata@dent.osaka-u.ac.jp (S.K.)
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SUMMARY

The arginine deiminase (ADI) pathway has been found in many kinds of bacteria and functions to supplement energy production and provide protection against acid stress. The Streptococcus pyogenes ADI pathway is upregulated upon exposure to various environmental stresses, including glucose starvation. However, there are several unclear points about the advantages to the organism for upregulating arginine catabolism. We show that the ADI pathway contributes to bacterial viability and pathogenesis under low-glucose conditions. S. pyogenes changes global gene expression, including upregulation of virulence genes, by catabolizing arginine. In a murine model of epidermal infection, S. pyogenes uses the ADI pathway to augment its pathogenicity by increasing the expression of virulence genes, including those encoding the exotoxins. We also find that arginine from stratum-corneum-derived filaggrin is a key substrate for the ADI pathway. In summary, arginine is a nutrient source that promotes the pathogenicity of S. pyogenes on the skin.

INTRODUCTION

One of the most important human bacterial pathogens of skin is Streptococcus pyogenes, which can produce superficial impetigo or more deep-seated cellulitis but also more severe invasive infections such as sepsis, necrotizing fasciitis, and streptococcal toxic shock syndrome (Cunningham, 2000; Walker et al., 2014). S. pyogenes typing based on M protein and T antigen (pilus major subunit) antigenicity (Falugi et al., 2008) confirms several serotypes are capable of causing severe infections, but in recent decades, one subclone of the M1 serotype, the globally disseminated clonal M1T1 clone (Chatellier et al., 2000), has persisted uninterrupted as the most frequently isolated S. pyogenes strains from both invasive and noninvasive infections (Lynskey et al., 2019; Walker et al., 2014).

Human skin is an inhospitable environment that includes the physical barrier of the stratum corneum (SC), an acidic surface pH, and the active synthesis of innate defense factors such as antimicrobial peptides, proteases, lysozymes, cytokines, and chemokines that together serve to recruit immune cells and prime adaptive immune responses (Cogen et al., 2008; Proksch, 2018). To successfully colonize or establish infection in the skin, pathogens must possess virulence determinants for evasion of these immune factors and also for acquisition of nutrients, whose availability the host may restrict under the concept of nutritional immunity. Elucidation of such bacterial metabolic pathways that are essential for in vivo survival can reveal unique targets for novel therapeutics.

The arginine deiminase (ADI) pathway is a metabolic pathway found in many kinds of bacteria that serves to supplement energy production and provide protection against acid stress in vitro (Abdelal, 1979; Cotter and Hill, 2003). The ADI pathway of S. pyogenes has been studied in a limited manner and determined to antagonize nitric oxide (NO) production by macrophages and contribute to the asymptomatic colonization of the murine vaginal mucosa (Cusumano et al., 2014). The S. pyogenes ADI pathway is negatively regulated by the following three virulence-related transcriptional control systems: the control
of virulence (CovRS) two-component gene regulatory system, catabolite control protein CcpA, and regulator gene of glucosyltransferase Rgg (Dmitriev et al., 2006; Shelburne et al., 2010). CovRS mediates a general stress response to changing temperature, pH, and osmolarity (Dalton and Scott, 2004); and CcpA and Rgg are directly linked to environmental glucose deprivation (Dmitriev et al., 2006; Shelburne et al., 2008). Although CovR deletion induces a several-fold increase of ADI pathway genes, CcpA or Rgg deletions markedly induce several-log-fold increases in the pathway (Dmitriev et al., 2006; Shelburne et al., 2010). These findings suggest that the ADI pathway is especially important to S. pyogenes under low-glucose conditions. Notably, the glucose concentration in the SC of skin is much lower than that present in blood (Sylvestre et al., 2010). In this study, we investigated whether the S. pyogenes ADI pathway contributes to the viability and pathogenesis of an M1T1 strain under low-glucose conditions and in the skin.

RESULTS

S. pyogenes ADI pathway contributes to its viability and virulence

The S. pyogenes ADI pathway is comprised of ArcA, B, C, and D (Figure S1A; Cusumano et al., 2014). ArcA, an arginine deiminase, is the first enzyme of the ADI pathway and catalyzes the irreversible hydrolysis of arginine to citrulline and ammonia. We constructed a markerless complete arcA deletion mutant (ΔarcA) by using a double crossover homologous recombination technique, preserving as a control a wild-type (WT) revertant strain (Wr) from the single crossover step back to the integrity of arcA. We first observed the time course of pH change of bacterial cultures grown under arginine-rich conditions, using phenol red as an indicator of elevated pH (Figure 1A). The WT S. pyogenes parent strain induced pH elevation in the culture medium beginning in stationary phase, whereas the S. pyogenes ΔarcA did not, confirming that a loss of arcA renders the bacterium’s arginine catabolism dysfunctional. Deletion of arcA led to a decrease in S. pyogenes viability during the decline phase of stationary growth in Todd-Hewitt broth supplemented with 0.2% yeast extract (THY broth) (Figure 1B). In contrast, the WT S. pyogenes strain exhibited a strong increase in arcA gene expression and ammonium ion production occurring during stationary phase following glucose starvation (Figures 1C, 1D, and 1E). Although a pH elevation was not detected during the stationary phase in THY broth, pH levels in phenol red broth supplemented with arginine were elevated above neutral pH for both the WT and Wr cultures (Figures S1B and S1C). Both WT and Wr showed the potential for long-term survival in neutral pH for at least 40 days during the decline phase, whereas the mutant strain lost viability after only 2 days (Figure S1B). These findings indicate that S. pyogenes neutralizes excess protons during stationary phase by synthesizing ammonia in an ADI-dependent manner, thus promoting long-term viability during the decline phase.

S. pyogenes cultured in THY broth experienced glucose starvation beginning in stationary phase. To investigate whether arginine catabolism contributed to S. pyogenes virulence phenotypes under such glucose-starved conditions, we prepared chemically defined medium (CDM; Table S1) without glucose, phenol red, or arginine. As a first surrogate marker of virulence, we evaluated S. pyogenes cytotoxicity against the cultured human keratinocyte cell line HaCaT. We supplemented 1,000 μM arginine in the CDM to match the physiological concentration present in human muscle tissue (Canepa et al., 2002). At 20 h after infection of HaCaT cells under arginine-supplemented conditions, WT and Wr showed a dose-dependent increase in cytotoxic potential compared to the ΔarcA mutant (Figures 1F and S1D), linking arginine catabolism to this virulence phenotype.

By metabolizing arginine, S. pyogenes acquires adenosine triphosphate (ATP) as an energy source, coupled to discharge of ammonium ion (Figure S1A). Intracellular acidification may affect the growth and cell viability of streptococci (Dashper and Reynolds, 2000). We probed the contribution of arginine catabolism to S. pyogenes intracellular pH and ATP levels under low-glucose conditions using CDM. No effects on the intracellular pH of S. pyogenes were observed 1 h, 5 h, and 15 h post-incubation in CDM (Figure S1E). Conversely, at 1 h and 5 h post-incubation, arginine catabolism contributed to the acquisition of ATP under the no-glucose condition (Figure 1G), which correlated with viability of S. pyogenes at 5 h and 15 h post-incubation in CDM (Figure 1H). S. pyogenes WT in CDM at 15 h had greater viability when cultured in the presence of HaCaT cells than in their absence (Figure 1H), suggesting that ADI-dependent S. pyogenes cytotoxicity induces release of nutrition from damaged keratinocytes, promoting S. pyogenes survival.
These results indicate that arginine catabolism contributes to S. pyogenes. We conducted RNA sequencing (RNA-seq) analysis of the S. pyogenes strains in CDM with arginine (Arg(+)) or without arginine (Arg(-)) to assess transcriptional consequences of altering the ADI pathway. Principal-component analysis (PCA) showed ΔarcA Arg(-), ΔarcA Arg(+), and WT Arg(-) samples clustered together, whereas WT Arg(+) samples positioned clearly apart from the others (Figure 2A). Differentially expressed genes (DEGs) from comparisons of the Arg(+) and Arg(-) groups were detected only in the WT S. pyogenes background and not with the ΔarcA (Figure 2B; Data S1 and S2). The cumulative results indicated that arginine-dependent transcriptome changes in S. pyogenes occurred only in the presence of an intact ADI pathway. A heatmap using selected characteristic genes showed that upregulated genes in WT Arg(+) samples included those encoding virulence factors, such as an enzyme for maturation of the cytolysin streptolysin S (sagB), another cytolysin streptolysin O (slo), nucleases (spod3), and NADase (nra), but also genes comprising the pyrimidine biosynthesis pathway (pyrD, pyrE, and pyrF) (Figure 2C). On the other hand, cell-division-associated genes (ftsH, ftsL, and ftsZ) and genes encoding F_{2}F_{1}-type ATP synthase (atpB–H) were downregulated in WT S. pyogenes in the presence of arginine.

The most well-studied S. pyogenes two-component signal transduction system is the cluster of virulence (Cov) intracellular responder (CovR)/extracellular sensor (CovS) CovRS, which influences 15%–20% of the S. pyogenes genome (Graham et al., 2002). CovS sensed environmental changes and transmitted to CovR by phosphorylation-dephosphorylation (Horstmann et al., 2002). Of particular interest, the covS gene was downregulated in WT S. pyogenes-sensing arginine (Figure 2C; Data S2). To investigate whether arginine catabolism influences CovR phosphorylation, we monitored phosphorylation of the regulator using a 5’-FLAG-CovR strain (Figure 2D). Exponential phase growth of S. pyogenes in THY broth showed low levels of CovR phosphorylation. For S. pyogenes within CDM, the WT Arg(+) sample tended to show a low level of CovR phosphorylation, but no significant difference was found by the densitometric analysis (Figure 2E).

ADI pathway contributes to the development of cutaneous lesions

The ADI-pathway-dependent bacterial virulence was canceled in Fig^{−/−} mice

Arginine is abundant in filamentin, an abundant protein within the SC of skin, and a filamentin knockout (Fig^{−/−}) mouse has a
markedly lower SC arginine level than a corresponding WT mouse (Kawasaki et al., 2012; Kubo et al., 2013). The degradation of filaggrin into amino acids occurs in the SC layers by host-derived enzymes, including caspase-14, calpain 1, and bleomycin hydrolase (Hoste et al., 2011). We hypothesized that S. pyogenes acquires arginine from filaggrin in the SC for nutritional requirements and its pathogenicity on the skin surface.

Figure 2. Effect of S. pyogenes arginine catabolism on transcriptome and phosphorylation of CovR
(A) Principal-component analysis (PCA) plot of fragments per kilobase of exon per million mapped fragments (FPKM) data from the RNA-seq dataset.
(B) Volcano plots comparing global gene expression patterns between WT Arg(-) and WT Arg(+) and between ΔarcA Arg(-) and ΔarcA Arg(+). Colored circles indicate significantly upregulated (red) and downregulated (blue) genes (absolute log2-fold change, >0.5; adjusted p < 0.2).
(C) Heatmap of up- or downregulated functions. FPKM values were used for the heatmap visualization. Red and blue indicate induced and repressed, respectively.
(D) Phosphorylation levels of CovR. CovR~P and CovR indicate phosphorylated CovR and non-phosphorylated CovR, respectively. Total protein serves as the loading control. THY, RNA samples from exponential phase of S. pyogenes in THY medium.
(E) Relative percentage of phosphorylated CovR. Error bars indicated the mean ± SE (n = 4). Arg(-), strains in CDM without arginine. Arg(+), strains in CDM with 1,000 μM arginine.
Figure 3. The roles of arginine catabolism for the virulence of *S. pyogenes* on the skin surface and in blood

(A) Murine model of epicutaneous infection and its timeline. Mice were epicutaneously infected with $2 \times 10^6$ CFU of *S. pyogenes*.

(B) Skin phenotype and histopathology at 3 days post-infection. Cutaneous tissues from infection sites were stained with H&E. Data shown are representative of at least three separate experiments.

(C) CFUs in skin lesions at 3 days post-infection. Data shown represent the mean ± SE (n = 8) and are representative of at least three independent experiments. **p < 0.01, *p < 0.05.

(legend continued on next page)
At baseline, it is difficult to appreciate any phenotypic differences between WT mice and Flg <sup>-/-</sup> mice (Figure 4A). However, following epicutaneous challenge assessed at 3 days post-infection, the previously observed difference of virulence between WT and ΔarcA disappeared in the skin of Flg <sup>-/-</sup> mice, as verified at three different challenge inocula (Figure 4B). These results were corroborated with similar histopathology of the skin lesions at the 3-day post-infection time point (Figure 4C). The ArcA protein is associated with the S. pyogenes bacterial cell surface (Henningham et al., 2012). To confirm that S. pyogenes expresses ArcA during infection on the skin surface, immunofluorescence staining was performed at 24 h post-infection. Strong signals for ArcA expression were seen only in the case of S. pyogenes WT on the skin surface of WT mice (Figures 4D and 4E). In Flg <sup>-/-</sup> mice, S. pyogenes did not appear to upregulate ArcA for arginine catabolism to achieve infection.

S. pyogenes showed different expression levels of covS, arcA, and slo genes on the skin surface 3 h after epicutaneous infection in WT compared to Flg <sup>-/-</sup> mice (Figure 5A). Because Flg <sup>-/-</sup> mouse skin exhibits enhanced permeability of SC as compared to WT mouse skin (Kawasaki et al., 2012), these transcriptional differences might reflect differences of nutritional or stress environments.

In intestinal epithelial cells, it is thought that caspase-1 contributes to both pyroptosis and apoptosis during an inflammation (Lei-Leston et al., 2017). To investigate whether S. pyogenes caused programmed cell death of skin epithelial cells, immunofluorescence staining of caspase-1 was performed 24 h post-epicutaneous infection. We saw a marked decrease of caspase-1 expression in epithelial cells infected with the ΔarcA in only WT mice (Figures 4F and 4G). S. pyogenes induces keratinocyte apoptosis by SLO-mediated membrane damage (Cywes Bentley et al., 2005). In S. pyogenes infection of HaCaT cells with CDM, an increase in released lactate dehydrogenase (LDH) in culture supernatants indicated that SLO contributed partially to arginine catabolism-dependent cytotoxicity (Figure 5A). Next, we determined whether pyroptosis or apoptosis was induced in infected epithelial cells by measuring interleukin-1β (IL-1β) release and DNA fragmentation by TUNEL staining. Because it was difficult to discriminate between pro-IL-1β and mature IL-1β by ELISA, we assessed signaling activity using HEK-Blue IL-1β reporter cells for quantifying pyroptosis. The secretion of mature IL-1β from HaCaT cells was enhanced in both WT and Δslo by arginine catabolism (Figure 5B), with SLO partially contributing to the pyroptosis phenotype, paralleling the cytolytic effect. On the other hand, in a TUNEL assay designed to detect apoptotic cells, arginine-catabolism-dependent apoptosis was not observed (Figure 5C). Taken together, S. pyogenes SLO contributes significantly to the pyroptosis of HaCaT cells induced by arginine catabolism, and there are other S. pyogenes factors that are likely involved in arginine-catabolism-independent pyroptosis.

**DISCUSSION**

The S. pyogenes ADI pathway is controlled by virulence-related metabolic regulators (Dmitriev et al., 2006; Shelburne et al., 2010) and is highly expressed in vivo (Graham et al., 2006; Hirose et al., 2019) and ex vivo (Graham et al., 2005; Shelburne et al., 2005). Here, we show that the S. pyogenes ADI pathway influences virulence factor expression and contributes to keratinocyte cytotoxicity under low-glucose conditions in vitro and subcutaneous infection in vivo. Our data support a model in which S. pyogenes uses arginine abundant in filaggrin of the SC, can secure nutrition, survive, and produce skin infection associated with local tissue destruction.

We found that S. pyogenes can survive for more than 40 days if it can maintain neutral pH by metabolizing arginine during the stationary phase. This result is consistent with a previous report that proved the long-term survival potential of S. pyogenes in neutral pH (Savic and McShan, 2012). The ability of the S. pyogenes ADI pathway to supplement energy production and provide protection against acid stress might greatly contribute to its viability in specialized environments such as skin.

Comparative transcriptome analysis reveals arginine-catabolism-dependent gene regulation under glucose-starvation conditions. Upregulated genes in the WT strain include genes in the pyrimidine biosynthesis pathway (pyrD, pyrE, and pyrF). In S. pyogenes, the ADI pathway cooperates with the pyrimidine biosynthesis pathway to acquire pyrimidine ribonucleotides and ATP (Hirose et al., 2019). Conversely, genes encoding F<sub>0</sub>F<sub>1</sub>-type ATP synths were downregulated in the WT strain compared to those in the ΔarcA mutant. Although it has been reported that the generation of ATP by the ADI pathway and a functional F<sub>0</sub>F<sub>1</sub>-type ATP synthase work in concert to adapt to acid stress (Cusumano and Caparon, 2015), it is speculated that S. pyogenes in CDM at neutral pH decreases the concomitant hydrolysis of ATP to ADP to reduce ATP consumption. Genes related to cell division (ftsH, ftsL, and ftsZ) were also downregulated in WT S. pyogenes, which might prioritize the expression of cytolytic virulence factors when there are not enough local nutrition sources to proliferate.

The deletion of covR induces the upregulation of virulence genes contained within the streptolysin S operon (sagABCDEFGHI) and the nga-slo operon and also shows downregulation of a dipeptide permease operon (dppABCD) (Shelburne et al., 2010). These
Figure 4. Arginine-catabolism-independent virulence of *S. pyogenes* on the skin surface of filaggrin knockout mice
(A) Skin phenotype and histology of both WT mice and *Fig*−/− mice.
(B) CFUs in skin lesions at 3 days post-infection. Data shown represent the mean ± SE (n = 10–12). **p < 0.01.

(legend continued on next page)
findings were mirrored in our results from RNA-seq analysis. Although CovR phosphorylation enhances DNA binding of CovR (Graham et al., 2002) and CovR affects the expression of 15%–20% of S. pyogenes genes (Horstmann et al., 2015), S. pyogenes arginine catabolism did not significantly influence CovR phosphorylation in our assay. Taken together, these results suggest that the main mechanism of S. pyogenes pathogenicity under glucose-depleted conditions may be dependent on acquiring ATP by catabolism of arginine.

Although transcript levels of the ADI operon were reduced in serotype M1 S. pyogenes (MGAS5005) after human blood exposure, temporal and mild upregulation of ADI operon were observed within 90 min of blood exposure (Graham et al., 2005). These investigators also reported that the deletion of the S. pyogenes covR regulator led to upregulation of the ADI operon in blood. These results partly contrast with our data. However, human blood is relatively poor in arginine but rich in glucose (Canepa et al., 2002; Sylvestre et al., 2019). Therefore, although CovRS might mediate some environmental signals in the blood and upregulate ADI operon expression, we speculate that arginine catabolism changes are not sufficient to drive pathogenesis of S. pyogenes in blood. In contrast, the ΔarcA mutant showed lower virulence than the WT S. pyogenes strain in a mouse soft-tissue infection model that was associated with localized necrosis in adjacent muscle. This difference in pathogenicity might be explained by high concentrations (approximately 1,000 μM) of arginine in muscle tissue (Canepa et al., 2002).

In a murine model of epicutaneous infection and in our RNA-seq analysis, S. pyogenes WT upregulated the arcA, slo, and sagA genes. High expression levels of these genes were reported in S. pyogenes isolated directly from mouse soft tissue infection (Graham et al., 2006) and a mouse model of necrotizing fasciitis (Hirose et al., 2019). Streptolysin S is involved in cellular injury, phagocytic resistance, and virulence in murine subcutaneous infection models (Datta et al., 2005; Humar et al., 2002). The upregulation of SLO has been correlated with a highly virulent S. pyogenes phenotype (Zhu et al., 2015). Our finding that ADI contributes to the expression of the sagA and slo genes might be important information for mitigating the pathogenicity of S. pyogenes.

The SC is the outermost layer of the epidermis and acts as the first line of structural defense against pathogens and toxins. Filaggrin, a major structural protein in the SC (Sandilands et al., 2009), contributes to the mechanical strength and integrity of the SC in vivo (Kawasaki et al., 2012), and filaggrin breakdown products form natural moisturizing factors that are believed to play a major role in SC hydration (Rawlings and Harding, 2004). Arginine is a major component of filaggrin-derived natural moisturizing factors (Kubo et al., 2013). In our epicutaneous infection model with Flg−/− mice, S. pyogenes might more easily penetrate to reach viable epidermal cells below the SC whereupon cytotoxic factors can allow the pathogen to secure nutrition from the viable host epidermal cells. Thus, ΔarcA could exert pathogenesis by using ADI-independent mechanisms on the skin surface of Flg−/− mice.

The SC of skin is also rich in lipids, such as ceramides, cholesterol, and free fatty acids (Elias and Schmuth, 2009), and bacterial infection of the skin also activates the host immune response (Cogen et al., 2008) and promotes acidic pH (Proksch, 2018). In our results, there were certain differences between our in vitro and in vivo findings that could not be explained based solely on S. pyogenes ADI pathway activity. Further experiments will be required to confirm whether other factors are involved in the pathogenesis of S. pyogenes and reveal more details about interactions between S. pyogenes and host skin tissue.

We revealed that S. pyogenes induced increased pyroptosis of HaCaT cells in a manner dependent on arginine catabolism, whereas almost no apoptosis was observed both dependently and independently of arginine catabolism. However, Cywes Bentley et al. (2005) reported that S. pyogenes SLO contributed to keratinocyte apoptosis. This discrepancy may be due to the difference of the keratinocyte cell line used or nutritional conditions. Because SLO was not fully responsible for the observed pyroptosis, further exploration will be needed to fully clarify the arginine-catabolism-dependent pyroptosis-inducing factors of S. pyogenes.

In summary, our findings suggest that S. pyogenes uses arginine from SC-derived filaggrin to adapt to glucose starvation on the skin surface. Despite the fact that arginine is a molecule that contributes to natural moisturizing of the skin, it can be simultaneously exploited by S. pyogenes that may metabolize arginine to promote its pathogenesis.

**STAR METHODS**

Detailed methods are provided in the on-line version of this paper and include the following:

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- **RESOURCE AVAILABILITY**
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  - Materials availability
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(C) Skin phenotype and histopathology at 3 days post-infection. Mice were epicutaneously infected with 2 × 10^7 CFU of S. pyogenes. Cutaneous tissues from infection sites were stained with H&E. Data shown are representative of at least three separate experiments.

(D) Representative microscopic images of immunofluorescence staining of S. pyogenes (red) and arginine deiminase, ArcA (green), on the skin surface at 24 h post-infection. Strong merge signals (yellow) were detected only in S. pyogenes WT on the skin surface of WT mice.

(E) Mean fluorescence intensity (MFI) of ArcA (minimum 10 bacteria per condition, n = 8). MFI was quantified using ImageJ. Average background fluorescence was subtracted from each value. Data represent mean ± SE. **p < 0.01.

(F) Representative microscopic images of immunofluorescence staining of S. pyogenes (red) on the skin surface and caspase-1 (green) in epidermis at 24 h post-infection. Weak signals of epithelial cells were shown only in ΔarcA-infected WT mice.

(G) The percentage of caspase-1-positive cells in epidermis (minimum 20 cells per condition, n = 8). Data represent mean ± SE. **p < 0.01. Flg−/−, filaggrin knockout mouse.
EXPERIMENTAL MODEL AND SUBJECT DETAILS
- Bacterial strains and culture conditions
- Construction of mutant strains
- Cell culture and media
- Murine model of epicutaneous and intravenous infections
- Mouse model of S. pyogenes necrotizing skin infection
- Mouse model of intraperitoneal infection
- Streptozotocin-induced diabetic mice

METHOD DETAILS
- Quantitative real-time PCR (qPCR)
- Measurement of glucose and ammonium ion concentrations
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- Measurement of intracellular pH
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- Blood bactericidal assay
- Immunofluorescence staining
- IL-1β signaling assay
- Apoptosis determination by Terminal transferase deoxyribo- nuclease end labeling (TUNEL) staining

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.celrep.2021.108924.

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AUTHOR CONTRIBUTIONS
Conceptualization, Y.H., M.Y., and S.K.; methodology, Y.H., M.Y., T.S., M.N., Y.M., H.K., S.U., M.H., and M.A.; software, D.O. and D.M.; investigation, Y.H., A.C., and R.H.Z.; formal analysis, Y.H. and T.H.; data curation, Y.H.; supervision, M.Y., V.N., and S.K.; visualization, T.H., D.O., and D.M.; funding acquisition, Y.H., M.Y., V.N., and S.K.; writing - original draft, Y.H.; writing - review & editing, M.Y., T.S., M.N., V.N., and S.K.; project administration, V.N. and S.K.

DECLARATION OF INTERESTS
The authors declare no conflict of interest.

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# STAR METHODS

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Donkey anti-Goat IgG Alexa Fluor 594 | Thermo Fisher Scientific | Cat# A32758, RRID:AB_2762828 |
| Donkey anti-Rabbit IgG H&L Alexa Fluor 488 | Abcam | Cat# ab150065, RRID:AB_2860569 |
| Goat polyclonal anti-\textit{S. pyogenes} carbohydrate | Abcam | Cat# ab9191, RRID:AB_307061 |
| Rabbit polyclonal anti-caspase-1 | GeneTex | Cat# GTX14368 |
| Bacterial and virus strains |        |            |
| \textit{Streptococcus pyogenes} M1T1 strain 5448 | Kansal et al., 2000 | GenBank: CP008776 |
| XL-10 Gold | Agilent Technologies | Cat# 200314 |
| Chemicals, peptides, and recombinant proteins |        |            |
| Carboxyfluorescein diacetate succinimidyl ester | Invitrogen | Cat# C1157 |
| Mutanolysin | Sigma Aldrich | Cat# M9901 |
| Staurosporine | Sigma Aldrich | Cat# S6942 |
| Streptozotocin | Adipogen | Cat# 50-464-382 |
| Critical commercial assays |        |            |
| ATP-bioluminescent assay Kinshiro | TOYO B-Net | Cat# LL100-1 |
| Click-IT Plus TUNEL Assay Kit (Alexa Fluor 488) | Thermo Fisher Scientific | Cat# C10617 |
| Glucose Colorimetric Assay Kit II | Biovision | Cat# K686 |
| LIVE/DEAD Viability/Cytotoxicity Kit | Thermo Fisher Scientific | Cat# L3224 |
| Deposited data |        |            |
| Raw data files for RNA-sequencing | DDBJ SRA | SRA: DRA009112 |
| Experimental models: Cell lines |        |            |
| HEK-Blue IL-1β reporter cells | InvivoGen | Cat# hkb-il1bv2 |
| Human: HaCaT cells | (Boukamp et al., 1988) | Human: HaCaT cells |
| Experimental models: Organisms/strains |        |            |
| Flaggrin-null mouse strain (B6.Cg-Fg < tm1 > ) | RIKEN BRC | RBRC05850 |
| Oligonucleotides |        |            |
| See Table S2 |        | N/A |
| Recombinant DNA |        |            |
| Plasmid: pSET4-ArcAKO | This paper | N/A |
| Plasmid: pSET4-5Flag-CovR | This paper | N/A |
| Plasmid: pQE30_arcA | This paper | N/A |
| Software and algorithms |        |            |
| CLC Genomics workbench v. 9.5.2 | Software | https://digitalinsights.qiagen.com/ja/qiagen-clc-genomics-workbench/ |
| ClustVis | Software | https://biit.cs.ut.ee/clustvis/ |
| GraphPad Prism7 | Software | https://www.graphpad.com/scientific-software/prism/ |
| iDEP.91 | Software | http://bioinformatics.sdstate.edu/idep/ |
| ImageJ | Software | https://imagej.nih.gov/ij/ |
| ImageQuant software | Software | http://www.cytivalifesciences.com/en/us/shop/protein-analysis/molecular-imaging-for-proteins/imaging-software/imagequant-ti-8-1-p-00110 |

(Continued on next page)
RESOURCE AVAILABILITY

Lead contact
Further information and requests for reagents may be directed to, and will be fulfilled by the corresponding author Yujiro Hirose (yujirohirose@dent.osaka-u.ac.jp).

Materials availability
This study did not generate new unique reagents.

Data and code availability
The accession number for the bacterial RNA-seq reads reported in this paper is SRA: DRA009112.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Bacterial strains and culture conditions
Streptococcus pyogenes M1T1 strain 5448 (GenBank: CP008776.1) was isolated from a patient with toxic shock syndrome and necrotizing fasciitis that is genetically representative of a globally disseminated clone associated with invasive S. pyogenes infections (Kansal et al., 2000). S. pyogenes strains were grown at 37°C in a screw-cap glass tube (Pyrex; Iwaki Glass, Tokyo, Japan) filled with Todd-Hewitt broth (BD Biosciences, San Jose, CA, USA) supplemented with 0.2% yeast extract (BD Bioscience) (THY broth) in an ambient atmosphere and standing cultures. To obtain cultures for experiments and observe pH change, overnight cultures of S. pyogenes were back diluted 1:50 into fresh THY broth or phenol red broth (Sigma Aldrich, St Louis, MO, USA) supplemented with 30 mM arginine. CFUs were determined by plating diluted samples on THY blood agar.

Escherichia coli strain XL-10 Gold (Agilent Technologies, Santa Clara, CA, USA) was used as a host for derivatives of plasmids pSET4s (Takamatsu et al., 2001) and pQE30 (QIAGEN, Hilden, Germany). E. coli strains were cultured in Luria-Bertani medium (Nacalai Tesque, Kyoto, Japan) at 37°C with agitation. For selection and maintenance of strains, antibiotics were added to the medium at the following concentrations: spectinomycin, 100 μg/mL for S. pyogenes and E. coli; carbenicillin, 100 μg/mL for E. coli.

Construction of mutant strains
An in-frame arcA deletion mutant (ΔarcA) and its revertant strain (Wr) with a background of strain 5448 (WT) were constructed using the pSET4s temperature-sensitive shuttle vector, as previously reported (Nakata et al., 2011). A pSET4-ArcAKO plasmid harboring the DNA fragment, in which upstream and downstream regions of arcA were linked by overlapping PCR, was electroporated into
strain 5448 and grown in the presence of spectinomycin. The plasmid was then integrated into the chromosome via first allelic replacement at 37°C, after which it was cultured at 28°C without antibiotics to induce the second allelic replacement. The deletion of arcA was confirmed by site-specific PCR using purified genomic DNA. Primers are listed in Table S2.

**Cell culture and media**

We used the immortal human keratinocyte line, HaCaT cells. HaCaT cells were cultured in Dulbecco’s Modified Eagle Media (DMEM, Cat#: 10-013-CV, Corning, NY, USA), with 10% Fetal Bovine Serum (Cat# 97068-085, VWR International LLC, Radnor, USA). The cell culture was maintained in a humidified 5% CO2 atmosphere at 37°C. The cells were cultured to around 70% confluence. To subculture cells, adherent cells were rinsed with PBS without calcium and magnesium, and detached by using trypsin/EDTA solution (0.05% trypsin, 0.53 m EDTA) for ~10 min, added fresh culture medium, centrifuged, resuspended cells in fresh culture medium, and dispensed into new culture vessels. For all experiments, freshly trypsinized cells were seeded at a density of ~1 × 10⁶ cells/cm² one day prior to bacterial infection.

**Murine model of epicutaneous and intravenous infections**

All mouse experiments were conducted in accordance with animal protocols approved by the Animal Care and Use Committee of Osaka University Graduate School of Dentistry (30-011-0) and University of California San Diego Institutional Animal Care and Use Committee (IACUC). The filaggrin null mouse strain (B6.Cg-Flg < tm1 > , RBRC05850) was provided by RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan.

Epicutaneous infections were performed using a previously reported with minor modifications (Nakamura et al., 2013; Sumitomo et al., 2018). Briefly, bacterial cultures during exponential phase were centrifuged, washed with and resuspended in PBS. Dorsal skin of C57BL/6 wild-type (wt) mice (6- to 7-week-old, both female and male; Japan SLC, Shizuoka, Japan) and the filaggrin null (Flg<sup>−/−</sup>) mice (Kawasaki et al., 2012) (6- to 7-week-old, both female and male) was depilated 2 days before infection. A bacterial suspension (5 × 10⁸-10⁹ CFU in 100 µL PBS) was placed on a 1 × 1 cm patch of sterile gauze, which is secured to the shaved skin with a transparent bio-occlusive dressing. At 3 hours post-infection, bacteria on the skin surface were collected by using a stainless dental scaler, then bacterial RNA was extracted and quantified by qPCR as described above. At 3 days post-infection, cutaneous tissue was excised for histopathologic analyses and assessment of bacterial burden. Cutaneous tissue samples were obtained and fixed with formalin, then embedded in paraffin, sectioned, and subjected to hematoxylin and eosin (HE) staining. Bacterial counts in cutaneous tissue homogenates were determined after plating serial dilutions, with those in the cutaneous tissue corrected for differences in tissue weight.

For intravenous infection, C57BL/6 wild-type mice (6- to 7-week-old, both female and male; Japan SLC) were intravenously infected with 2 × 10⁶ CFU of S. pyogenes during exponential phase, and survival was monitored for 14 days.

**Mouse model of S. pyogenes necrotizing skin infection**

Invasiveness of S. pyogenes in mouse skin was measured by modification of a previously described S. pyogenes infection model (Nizet et al., 2001). All mouse experiments were conducted in accordance with animal protocols approved by the Animal Care and Use Committee of Osaka University Graduate School of Dentistry (30-011-0). The CD-1 (Slc: ICR) mice (6 weeks old, female; Japan SLC, Shizuoka, Japan) were shaved and hair removed by chemical depilation (Veet, Oxy Reckit Benckiser, Chartes, France). S. pyogenes were cultured within CDM, bacterial cultures during exponential phase (OD<sub>600</sub> = 0.5-0.6), and adjusted 1 × 10⁷ CFU in 100 µL PBS was placed on a 1 × 1 cm patch of sterile gauze, which is secured to the shaved skin with a transparent bio-occlusive dressing. At 3 hours post-infection, bacteria on the skin surface were collected by using a stainless dental scaler, then bacterial RNA was extracted and quantified by qPCR as described above. At 3 days post-infection, cutaneous tissue was excised for histopathologic analyses and assessment of bacterial burden. Cutaneous tissue samples were obtained and fixed with formalin, then embedded in paraffin, sectioned, and subjected to hematoxylin and eosin (HE) staining. Bacterial counts in cutaneous tissue homogenates were determined after plating serial dilutions, with those in the cutaneous tissue corrected for differences in tissue weight.

**Mouse model of intraperitoneal infection**

Intraperitoneal infections were performed using a previously reported method with minor modifications (Valdes et al., 2016). C57BL/6 wild-type (wt) mice (6- to 7-week-old, both female and male; Japan SLC, Shizuoka, Japan) and the filaggrin null (Flg<sup>−/−</sup>) mice (Kawasaki et al., 2012) (6- to 7-week-old, both female and male) were intraperitoneally injected with 2.5 × 10⁶ CFU in 100 µL of PBS. Mouse survival was monitored for 14 days.

**Streptozotocin-induced diabetic mice**

To induce diabetes mellitus, C57BL/6 male mice (4-week-old) were injected i.p. with streptozotocin (Adipogen, San Diego, CA, USA) at 80 mg/kg/dose in 200 µL of 0.1 M citrate buffer daily for 4 days (Patras et al., 2020). Control mice received 4 daily treatments of 200 µL of 0.1 M citrate buffer. Mice were weighed weekly thereafter. The concentration of blood glucose in 7-week-old mice was determined 24 h prior to infection. Sample glucose was determined using an AimStrip Plus blood glucose meter kit (Germaine Labs, Indianapolis, IN, USA).

**METHOD DETAILS**

**Quantitative real-time PCR (qPCR)**

Bacterial cultures during exponential phase (OD<sub>600</sub> = 0.5-0.6), early stationary phase (OD<sub>600</sub> = 1.2), or decline phase (overnight culture) were centrifuged and immediately placed into RNAProtect Bacteria Reagent (QIAGEN) prior to RNA isolation. In the RNA isolation from S. pyogenes cultured within CDM, bacterial cultures during exponential phase (OD<sub>600</sub> = 0.5-0.6) were centrifuged,
resuspended into CDM, incubated in a screw cap glass tube (Pyrex; Iwaki Glass, Tokyo, Japan) for 1 h at 37°C, and immediately placed into RNAprotect Bacteria Reagent. *S. pyogenes* was resuspended into lysing Matrix B microtubes containing 0.1-mm silica spheres (Qbiogene, Carlsbad, CA, USA) with RLT lysis buffer (RNeasy Mini Kit; Qiagen), and homogenized at 8,500 rpm for 60 s using the MagNA Lyser (Roche Molecular Diagnostics, Mannheim, Germany). RNA was isolated from the lysate with RNeasy Mini Kit according to the manufacturer’s guidelines, and then cDNA was synthesized using a Superscript VILO cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA). Real-time reverse transcription PCR analysis was performed using a StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA, USA) and TOYO SYBR green RT-PCR master mix kit (Toyobo Life Science, Osaka, Japan). Data for 16S rRNA or rpOB were used as the internal control. Primers used for qPCR are listed in Table S2.

### Measurement of glucose and ammonium ion concentrations

Culture supernatant at each growth phase obtained from *S. pyogenes* WT and ΔarcA cultured in THY broth was filtered through a 0.22 μm membrane, then and directly analyzed with BioProfile® FLEX2 analyzer following manufacturer’s instruction (Nova Biomedical, Inc., Waltham, MA, USA).

### Measurement of extracellular pH

For phenol red broth (Sigma Aldrich, St Louis, MO, USA) supplemented with 30 mM arginine, culture supernatant at each point was measured the absorbance at 550 nm. A calibration curve was determined in phenol red broth which adjusted to pH values ranging from 4 to 10. The pH values were assessed up to 40 days in the sample which included surviving bacteria. For THY broth, culture supernatant at each point was supplemented with 5 mg/mL phenol red and the absorbance was measured at 550 nm. A calibration curve was determined in THY broth which was supplemented with 5 mg/mL phenol red and adjusted to pH values ranging from 4 to 10.

### Measurement of intracellular pH

The cytosolic pH of *S. pyogenes* was determined based on the previously described fluorescent probe method (Do et al., 2019). Briefly, *S. pyogenes* grown to log phase of growth in THY broth were centrifugated, washed in 150 mM NaCl, and resuspended in 50 mM HEPES buffer (pH 8.0). The cells were then incubated for 20 min at 37°C in the presence of 10 μM carboxyfluorescein diacetate succinimidyl ester (CFDA-SE, Invitrogen, Grand Island, NY, USA). CFDA-SE is hydrolyzed to carboxyfluorescein succinimidyl ester (CFSE) in the cell and subsequently conjugated to aliphatic amines of the intracellular proteins. After incubation, cells were washed and suspended in 50 mM potassium phosphate buffer (pH 7.5). To eliminate nonconjugated CFSE, cells were incubated with 10 mM glucose for 30 min at 30°C. Subsequently, *S. pyogenes* were washed, and suspended, and incubated in CDM at 37°C within a screw-cap glass tube. At 1 h, 5 h, and 15 h post-incubation, fluorescence intensities were determined with an excitation spectrum of 400-500 nm wavelength range that includes excitation wavelengths 490 nm (pH-sensitive) and 435 nm (pH-insensitive) (Spark 10M; TEKAN, Männedorf, Switzerland). Emission was determined at 520 nm. The ratio of the emission resulting from excitation at 490 and 435 nm obtained for both cell suspension (C) and filtrate (F) was calculated as R 490/435 = (C490 - F490)/C435 - F435. A calibration curve was determined in CDM adjusted to pH values ranging from 5.5 to 8.0 and a cubic equation for the ratio value was determined. Intracellular pH values of *S. pyogenes* were calculated using the cubic equation from the calibration curve.

### Infection of HaCaT cells with *S. pyogenes*

The composition of Chemically Defined Medium (CDM) is shown in Table S1. *S. pyogenes* in log phase growth in THY broth were centrifuged and resuspended into CDM supplemented with or without 1000 μM arginine. HaCaT cells were infected with *S. pyogenes* (MOI = 500). At 20 h post-incubation, supernatants were collected by centrifugation and analyzed for cytotoxicity assays, IL-1β signaling assay, and cells were used for the apoptosis determination.

### Cytotoxicity assays

Cell viability was assessed using the LIVE/DEAD® Viability/Cytotoxicity Kit (Thermo Fisher Scientific). LDH activity in the culture supernatant was measured by using LDH Assay Kit-WST (Dojindo, Kumamoto, Japan). At 1 h, 5 h, and 15 h post-infection, the bacterial count in CDM with cultured HaCaT cells was evaluated by combining CFUs from supernatant and those from associated with HaCaT cells. To determine bacterial association, HaCaT cells were harvested with PBS containing 0.05% trypsin and 0.025% Triton X-100.

### Measurement of intracellular ATP levels

At 1 h, 5 h, and 15 h post-incubation in CDM, the intracellular ATP levels of *S. pyogenes* were also evaluated by an ATP-bioluminescent assay using Kinoshio (TOYO B-Net, Tokyo, Japan) according to manufacturer’s instructions. Briefly, ATP extractant solution was added to equal amount of the mixture of CDM and *S. pyogenes*. After the incubation for 10 s at room temperature, 100 μL samples were mixed with equal amount of bioluminescent reagent, and bioluminescence was measured with a luminometer (Infinite 200 Pro multiplate reader, TEKAN, Männedorf, Switzerland), immediately. After establishing of the calibration curve, the ATP concentrations in the samples were determined, and the percentage of ATP levels for *S. pyogenes* at 0 h post-incubation was calculated.
RNA-seq and data analysis

Bacterial cultures during exponential phase were centrifuged, resuspended into CDM, and incubated in a screw-cap glass tube (Pyrex; Iwaki Glass, Tokyo, Japan) for 1 h at 37°C. RNA samples of S. pyogenes were obtained after incubation, as described above. RNA integrity was assessed using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). For RNA-seq, bacterial RNA was treated for rRNA removal using a Ribo-Zero rRNA removal kit (Illumina Inc., San Diego, CA, USA). RNA-seq libraries were created using a TruSeq RNA Sample Prep kit, v2 (Illumina Inc.), according to the manufacturer’s recommendations. Libraries were sequenced using Illumina HiSeq 2500 systems, with 75-bp single-end reads. RNA-seq reads were mapped against the S. pyogenes strain 5448 genome using the commercially available CLC Genomics workbench, v. 9.5.2 (CLC Bio, Aarhus, Denmark). Global analyses of RNA-seq expression data were performed using IDEP (Ge et al., 2018), with the FPKM value of each sample. We classified the differentially expressed genes (DEGs) into functional categories based on the bacterial bioinformatics database and analysis resource PATRIC (Wattam et al., 2017). The heatmap was visualized by use of the web tool ClustVis (Metsalu and Vilo, 2015) with default parameters.

Phos-tag western blotting for the detection of phosphorylated CovR

For this experiment, we constructed in-frame 5′-3xflag-tagged covR insertion mutants (5′Flag-CovR) of WT and ΔarcA. Utilizing strain S. pyogenes 5448 genome DNA as a template, two other DNA fragments were amplified using primer sets 5′Flag-CovRF1 and 5′Flag-CovRR1, or 5′Flag-CovRF1 and 5′Flag-CovRR1 (Table S2). Then, two fragments were PCR-linked, and a fragment encoding 5′-3xflag-tagged covR was created. Finally, a pSET4-5′Flag-CovR plasmid harboring 5′-3xflag-tagged covR was transformed to generate 5′Flag-CovR strain, as described above.

Bacterial cultures during exponential phase were centrifuged, resuspended into CDM, and incubated in a screw-cap glass tube for 1 h at 37°C. To extract total protein from S. pyogenes, we prepared our original lysis buffer which contains 10 U mutanolysin (Sigma Aldrich), Complete, EDTA (+) Protease Inhibitor Cocktail (Roche Molecular Diagnostic, Pleasanton, CA, USA), and PhosSTOP (Roche Molecular Diagnostic) in PBS. Bacterial cultures in THY broth during exponential phase or incubated S. pyogenes in CDM were centrifuged, and resuspended into lysing Matrix B microtubes containing 0.1-mm silica spheres with our original lysis buffer, and homogenized at 6,500 rpm for 30 s using the MagNA Lyser (Roche Molecular Diagnostic). The lysates were centrifuged, and the supernatants were applied to SDS-PAGE (Phos-tag SuperSep Phos-tag Gels; Wako Pure Chemical Industries, Osaka, Japan). The gel was transferred onto the Immobilon-FL PVDF (Millipore, Billerica, MA, USA) and phosphorylated CovR was detected by Anti-DDDDK-tag mAb-Alexa Fluor® 488 (MBL, Nagoya, Japan). Labeled proteins were visualized using Amersham Typhoon RGB Biomolecular Imager (Amersham Biosciences-GE Healthcare, Piscataway, NJ, USA). Relative percentage of phosphorylated CovR were calculated using ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA).

Detection of glucose concentration on the skin

At 48 h after depilation of dorsal skin of mice, a cup was placed on the skin and 100 μL PBS was injected. Then the skin was scratched by disposable inoculating loops and the sample was collected. Glucose concentration was measured with Glucose Colorimetric Assay Kit II (Biovision, Milpitas, CA, USA) following manufacturer’s instruction.

Blood bactericidal assay

Heparinized mouse blood (190 μL) and exponential phase bacteria (1.5 × 10⁶ CFU in 10 μL of PBS) were mixed in 96-well plates and incubated at 37°C in 5% CO₂ for 1, 2, or 3 hours. Viable cell counts were determined by plating diluted samples on THY blood agar. At 3 hours post mixing, bacterial RNA in blood were also isolated for qPCR. Blood samples were mixed with the component of RNAProtect® Animal Blood Tubes (QIAGEN), and centrifuged. Pellets were placed in lysing Matrix D microtubes containing 1.4-mm silica spheres (Qbiogene) with RLT lysis buffer (RNeasy kit; QIAGEN) and homogenized at 6,500 rpm for 45 s using a MagNA lyser. The lysate was centrifuged, and the obtained pellet was resuspended in lysing Matrix D microtubes containing 0.1-mm silica spheres (Qbiogene) with the RLT lysis buffer and homogenized at 6,500 rpm for 60 s using the MagNA lyser. The final lysate was centrifuged and bacterial RNA was isolated from the collected supernatant with a RNeasy kit, according to the manufacturer’s guidelines.

Immunofluorescence staining

Paraffin sections of cutaneous tissues of non-infected mice were subjected to immunofluorescence staining to detect filaggrin of mice. Following deparaffinization, sections in a 10 mM sodium citrate solution (pH 6.0) were heated for 5 min in a pressure cooker to retrieve the antigens.

Paraffin sections of cutaneous tissues at 24 hours post-infection were subjected to immunofluorescence staining to detect S. pyogenes, bacterial ArcA, and caspase-1 of host cells. ArcA was detected with rabbit antiserum against recombinant ArcA proteins which were purified from pQE30-ArcA transformed XL10-Gold by using Ni-NTA resin. Following deparaffinization, sections in a 10 mM sodium citrate solution (pH 6.0) were heated for 5 min in a pressure cooker. To visualize S. pyogenes and bacterial ArcA, goat polyclonal antibody to S. pyogenes carbohydrate (1/100; Abcam, Cambridge, MA, USA) and rabbit antiserum against recombinant ArcA (1/100) were applied after blocking with PBS containing 2% normal donkey serum. To visualize S. pyogenes and caspase-1 of host cells, goat polyclonal antibody to S. pyogenes carbohydrate (1/100) and rabbit polyclonal antibody to caspase-1 (1/100;
GeneTex, Irvine, CA, USA) were applied after blocking with PBS containing 2% normal donkey serum. Then, to visualize both of them, donkey anti-Goat IgG Alexa Fluor 594 (1/200; Thermo Fisher Scientific), and donkey anti-Rabbit IgG H&L Alexa Fluor 488 (1/200; Abcam) were applied as the secondary antibody.

Finally, all sections were mounted with ProLong Gold (Thermo Fisher Scientific). Stained tissue sections were examined with a Keyence microscope (Keyence Japan, Tokyo, Japan).

**IL-1β signaling assay**

Stably transfected HEK-Blue IL-1β reporter cells (InvivoGen, San Diego, CA, USA) (40,000 cells per well in 96-well plates), were stimulated at 37°C in 5% CO2 with 50 μL of supernatants from infected HaCaT cells. After 18 h stimulation, supernatants from the HEK-Blue cells were analyzed for secreted alkaline phosphatase activity by the addition of 50 μL of supernatants onto 150 μL of Quanti-Blue reagent (Invivogen) and monitoring the optical density at 620 nm via EnSpire plate reader (PerkinElmer).

**Apoptosis determination by Terminal transferase deoxytidyl uridine end labeling (TUNEL) staining**

At 20 h post-incubation, detection of apoptosis by TUNEL was performed using Click-iT Plus TUNEL Assay Kit (Alexa Fluor 488) (Thermo Fisher Scientific, Waltham, MA, USA) following manufacturer’s instruction. HaCaT cells were cultured and infected on pre-coated poly-L-lysine-chamber slide (Nunc Lab-Tek II Chamber Slide System) (Thermo Fisher Scientific). As a control, apoptosis of HaCaT cells were induced by treating with 0.5 μM staurosporine for 4 hours to induce apoptosis. Coverslips were mounted on slide glasses with ProLong Gold Antifade Reagent with DAPI (Thermo Fisher Scientific). The number of TUNEL-positive and DAPI-stained nuclei were determined and the apoptosis percentage was expressed as the ratio between TUNEL-positive and DAPI-stained nuclei. Six fields per condition (100 cells each) were observed. Cells were visualized using a Zeiss Axio Observer.D1 fluorescence microscope.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Statistical analysis was performed using GraphPad Prism version 7.0 (GraphPad Software Inc., La Jolla, CA, USA). Kruskal-Wallis test with Dunn’s post hoc test was used for multiple comparisons. Differences between groups were analyzed using a Mann-Whitney U test. A Mouse survival was analyzed with a log-rank test. Sample sizes and p values are indicated in figure legends.
Supplemental information

*Streptococcus pyogenes* upregulates arginine catabolism to exert its pathogenesis on the skin surface

Yujiro Hirose, Masaya Yamaguchi, Tomoko Sumitomo, Masanobu Nakata, Tomoki Hanada, Daisuke Okuzaki, Daisuke Motooka, Yasushi Mori, Hiroshi Kawasaki, Alison Coady, Satoshi Uchiyama, Masanobu Hiraoka, Raymond H. Zurich, Masayuki Amagai, Victor Nizet, and Shigetada Kawabata
Figure S1. Effects of arginine catabolism on extracellular or intracellular pH, and the cytotoxicity. Related to Figures 1 and 2. (A) Arginine catabolism in *S. pyogenes*. Through the multienzyme pathway, arginine is transported into the cell via the antiporter ArcD and catabolized by ArcA, ArcB, and ArcC to produce one molecule of carbon dioxide, one molecule of ATP, and two molecules of ammonia. ArcA,
arginine deiminase; ArcB, ornithine carbamoyltransferase; ArcC, carbamate kinase; ArcD, arginine/ornithine antiporter. (B) Relationship between pH level of bacterial culture and bacterial viability. A picture indicates pH levels of bacterial cultures in phenol red broth supplemented with 30 mM arginine at 24 h post-incubation. (C) Temporal pH change of bacterial cultures in THY broth. (D) Arginine catabolism-dependent cytotoxicity of S. pyogenes against HaCaT cells in a dose-dependent manner. Means ± S.E. (n = 4) are shown. Differences between groups were analyzed using a Mann-Whitney U test. **p < 0.01. (E) Intracellular pH values of S. pyogenes in CDM. Arg(-), strains in CDM without arginine. Arg(+), strains in CDM with 1000 μM arginine.
Figure S2. The virulence of S. pyogenes strains in other infection model, and arginine- or glucose-dependent up-regulation of slo gene expression levels of S. pyogenes. Related to Figures 3 and 4. (A) Mouse model of S. pyogenes necrotizing skin infection. Error bars indicated the mean ± S.E (n = 16). Statistical differences between groups were analyzed using a Kruskal-Wallis test with Dunn's post hoc test. **p < 0.01. (B) Mouse model of intraperitoneal infection (n = 10). Flg⁻/⁻, filaggrin knock-out mice. (C, D, E) Arginine catabolism independent virulence on the skin of diabetic mice. (C) An image of the sample collection for measuring the glucose concentration on the skin. (D)
glucose concentration on the skin both wild-type (wt) mice and diabetic mice (n = 10). (E) CFUs in skin lesions at 3 days post-infection in murine model of epicutaneous infection (n = 5). Error bars indicated the mean ± S.E. Statistical differences between groups were analyzed using a Mann-Whitney U test. **p < 0.01. (F, G) Arginine- or glucose- dependent up-regulation of slo gene expression levels of S. pyogenes. qRT-PCR was conducted by using RNA samples cultured in CDM for 1 h. Arginine (+), strains in CDM with 1000 μM arginine. Glucose (+), strains in CDM with 1000 μM glucose. The 16S rRNA was used as the internal control. Data from three independent qRT-PCR assays, each performed in triplicate, were pooled and normalized. Vertical lines represent the mean ± S.E. (n = 9). **p < 0.01. *p < 0.05.
Figure S3. The expression of transporter genes of *S. pyogenes* during epicutaneous infection. Related to Figure 3. qRT-PCR was conducted by using RNA samples which were collected from wild-type mice skin surface at 3 hours post-infection. Figure shows the gene expression of WT as compared to that in ΔarcA. In our RNA-seq data *in vitro*, 4 genes associated with phosphotransferase system (PTS) were upregulated in WT strain under arginine-rich condition, including mannose/fructose/sorbose family IID component (*ptsD*), mannose/fructose/sorbose family IIA component (*SP5448_05675*), cellobiose-specific IIB component (*SP5448_08850*), cellobiose-specific IIA component (*SP5448_08855*). Therefore, we evaluated the expression of transporter genes at 3 hours post-infection. The 16S rRNA was used as the internal control. Data from three independent qRT-PCR assays, each performed in triplicate, were pooled and normalized. Vertical lines represent the mean + S.E. (n = 9).
Figure S4. The expression of *covS*, *slo*, and *arcA* genes of *S. pyogenes* during epicutaneous infection. Related to Figures 3 and 4. qRT-PCR was conducted by using RNA samples which were collected from wild-type (wt) or filaggrin knock-out (*Flg*⁻⁻) mice skin surface at 3 hours after epicutaneous infection. Data shown are log₂-fold expression normalized to *rpoB* transcript levels. Shelburne et al. used *proS* transcript levels for normalization relative to a housekeeping gene (Shelburne et al., 2008 DOI: 10.1073/pnas.0711767105). However, our RNA-seq results indicate *proS* transcript levels were significantly downregulated dependently of arginine-catabolism. Therefore, *rpoB* transcript levels were used for normalization. Data from two independent qRT-PCR assays, each performed in triplicate, were pooled and normalized. Vertical lines represent the mean ± S.E. (n = 6). Statistical differences between groups were analyzed using a Mann-Whitney *U* test. **p < 0.01.
Figure S5. Involvement of Streptolysin O (SLO) in arginine-dependent cytotoxicity. Related to Figure 4. A-D, In CDM supplemented with or without 1000μM arginine, HaCaT cells were co-incubated with S. pyogenes WT or Δslo mutant at MOI 500 for 20 h. (A) Detection of LDH in culture supernatants. (B) Quantification of mature IL-1β in culture supernatants for detecting pyroptosis. (C) TUNEL reaction for detecting apoptosis. TUNEL-positive and DAPI-stained cells are indicated green and blue, respectively. The percentage of TUNEL-positive cells was calculated as the ratio between TUNEL-positive and DAPI-stained nuclei ×100. Representative data obtained from at least 3 independent experiments are shown. Vertical lines represent the mean ± S.E. (n = 6). Statistical differences between groups were analyzed using a Mann-Whitney U test. **p < 0.01. **p < 0.01.
Table S1: Composition of the chemically defined medium. Related to Figures 1 and 2

|                | Effective chemical       | Amount (mg/L) |
|----------------|--------------------------|---------------|
| **Amino acids**|                          |               |
| L-Arginine     |                          | 0.00          |
| L-Cystine      |                          | 48.34         |
| L-Glutamine    |                          | 584.00        |
| Glycine        |                          | 30.00         |
| L-Histidine HCl H2O |                  | 42.00         |
| L-Isoleucine   |                          | 105.00        |
| L-Leucine      |                          | 105.00        |
| L-Lysine HCl   |                          | 146.00        |
| L-Methionine   |                          | 30.00         |
| L-Phenylalanine|                          | 66.00         |
| L-Serine       |                          | 42.00         |
| L-Threonine    |                          | 95.00         |
| L-Tryptophan   |                          | 16.00         |
| L-Tryptophan   |                          | 16.00         |
| L-Tyrosine     |                          | 71.59         |
| L-Valine       |                          | 94.00         |
| **Vitamins**   |                          |               |
| D-1/2Ca Pantothenate |                      | 4.00          |
| Choline Chloride  |                          | 4.00          |
| Folic Acid     |                          | 4.00          |
| i-Inositol     |                          | 7.20          |
| Niacinamide    |                          | 4.00          |
| Pyridoxine HCl |                          | 4.00          |
| Riboflavin     |                          | 0.40          |
| Thiamine HCl   |                          | 4.00          |
| **Inorganic components** |         |               |
| CaCl2          |                          | 200.00        |
| KCl            |                          | 400.00        |
| MgSO4          |                          | 97.67         |
| NaCl           |                          | 6400.00       |
| NaHCO3         |                          | 3700.00       |
| NaH2PO4        |                          | 108.70        |
| **Trace elements** |                        |               |
| Fe(NO3)3 9H2O |                          | 0.10          |
Table S2. Primers used in this study. Related to Figures 1, 2, and 3.

| Primer          | Sequence (5'-3')                                                                 | Purpose                                      |
|-----------------|--------------------------------------------------------------------------------|----------------------------------------------|
| ArcAKOF1        | GTTAAGCTTTGTTGAGGCTCC                                                          | Construction of pSET4-ArcAKO                  |
| ArcAKOR1        | AAGAGATATCTCTTTCTAATTGG                                                       | Construction of pSET4-ArcAKO                  |
| ArcAKOF2        | TAAATGATCTGAAGGTGTTAT                                                         | Construction of pSET4-ArcAKO                  |
| ArcAKOR2        | AAGGATCCATGAGGCAACACC                                                        | Construction of pSET4-ArcAKO                  |
| ArcAKOcheckF    | AGAGATGAGGAGAAGGTGATT                                                        | Confirmation of arcA deletion                 |
| ArcAKOcheckR    | AAGGATAGACGACGTCACAGAAGAAG                                                   | Confirmation of arcA deletion                 |
| 5'Flag-CovRF1   | CGCGGATCCGGTTCTCTGTTG                                                         | Construction of pSET4-5'Flag-CovR            |
| 5'Flag-CovRR1   | CTTGTTCAATCTCATCCTTTGTATCAATGTATCTATTATAATCACCCTATCTGCTTTATTCGCACTATGCCCTTTATCC | Construction of pSET4-5'Flag-CovR            |
| 5'Flag-CovRF2   | GACTCAAAAGACCATGACGGTGTTTAAAGATGTCATGGCATGCGATTAACAGGATGACGACTGAAGACAAAGAAAAATTTAATTATGG | Construction of pSET4-5'Flag-CovR            |
| 5'Flag-CovRR2   | CCGAATTCCGGCGAGAAGGTGAGGCAAGAAG                                            | Construction of pSET4-5'Flag-CovR            |
| 5'FlagCovRCheckF| GACCGTGATTATAAAAGATCATG                                                       | Confirmation of 5'Flag-CovR                  |
| 5'FlagCovRCheckR| CATGATTGCAAGCAGCATG                                                         | Confirmation of 5'Flag-CovR                  |
| 5448arcAF       | GGTTGCGAAGAGGTGCTATG                                                         | qPCR of arcA                                 |
| 5448arcAR       | AAGTTCGTCGCCACCTTCAAT                                                        | qPCR of arcA                                 |
| 5448sagAF       | TGAGATTACCCCTCCACACGCAAGAAG                                                 | qPCR of sagA                                 |
| 5448sagAR       | CTCTCGGAGGCTGCTGTT                                                          | qPCR of sagA                                 |
| 5448sloF        | ACCTATCCAGAAGCCCTGCA                                                        | qPCR of slo                                  |
| 5448sloR        | CTACCAGCTGCTGGTTTTC                                                         | qPCR of slo                                  |
| 5448speBF       | GGCAGGACATGCGCTTTG                                                          | qPCR of speB                                 |
| 5448speBR       | TCCACCCCACCCCAGTGA                                                         | qPCR of speB                                 |
| 544816SrRNAF    | GTTTCAACCTTGGGCTGTTG                                                        | qPCR of 16SrRNA                              |
| 544816SrRNAR    | GGGTGTAGTGCGCGAGCTA                                                        | qPCR of 16SrRNA                              |
| 5448rpoBF       | CTGCCAGGAGGAGCAAAAA                                                        | qPCR of rpoB                                 |
| 5448rpoBR       | CATCAAAGAAAACCCGGCAATC                                                       | qPCR of rpoB                                 |
| 5448covSR       | TTGGCTACTAGTGGTTGAGTTATTTTGG                                                | qPCR of covS                                 |
| 5448ptsDF       | GCTCAATGACGGCTCCTCAAT                                                        | qPCR of ptsD                                 |
| 5448ptsDR       | CTGCTCTTTGGGACACCTTTC                                                        | qPCR of ptsD                                 |
| 544808850F      | AAGGCTATGCTCAAAGGGAAA                                                      | qPCR of SP5448_08850                         |
| 544808850R      | GCCTAAAGAATGCGCCATTCA                                                       | qPCR of SP5448_08850                         |
| 544808855F      | CTGGCAAGAAGAGCTATGTTG                                                       | qPCR of SP5448_08855                         |
| 544808855R      | CGTCAAAAGTGGATCTGAGT                                                         | qPCR of SP5448_08855                         |
| 544805675F      | AGCAGATGACGGGCGCTTAT                                                        | qPCR of SP5448_05675                         |
| 544805675R      | AGGGTACCATTTTTCTGATCA                                                       | qPCR of SP5448_05675                         |
| arcA_pQE30_F1   | CACATACCATCATTGCGTCGAGCAACACACAAATCATG                                    | Construction of pQE30-ArcA                   |
| arcA_pQE30_R1   | CAAGCTCAGCTAATTTTTATATCATTGACGTTCAATG                                      | Construction of pQE30-ArcA                   |
| pQE30_arcA_F1   | CTTTTCGAGTGAGCCTGAGTGTGGATGATGGCATGACGATCCTT                                 | Construction of pQE30-ArcA                   |
| pQE30_arcA_R1   | TGTGTGAGGACAGCAGTGGTGTGGATGATGGCATGACGATCCT                                 | Construction of pQE30-ArcA                   |