Differential Release and Distribution of Nod1 and Nod2 Immunostimulatory Molecules among Bacterial Species and Environments*

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Nod1 and Nod2 are intracellular proteins that are involved in recognition of bacterial molecules and their genetic variations have been linked to several inflammatory diseases that are strongly affected by environmental factors. However, the distribution of Nod1- and Nod2-stimulatory molecules in different bacterial species and environments is unknown. Here we established a quantitative bioassay to screen and characterize Nod1- and Nod2-stimulatory activities in different environmental sites and bacterial species. Using this system, we found that common bacterial species present in the body and in the environment possess the highest Nod1-stimulatory activity and bacterial species. Unlike other immunostimulatory molecules, the higher level of Nod1-stimulatory activity was found in soil bacteria. Several Bacillus species were identified to possess the highest Nod1-stimulatory activity among soil bacteria. Unlike other immunostimulatory molecules, the higher level of Nod1-stimulatory activity was found in the culture supernatant and not in extracts from whole cell bacteria. Nod1-stimulatory molecules were highly stable at extreme pH and boiling conditions and were synthesized in an amidas- and sltY-independent manner. These results suggest a novel mechanism by which bacteria present in the environment stimulate the host immune system through Nod1.

Animals including humans are always exposed to many bacterial species present in the body and in the environment. Some of these microorganisms are pathogens that can potentially cause disease, whereas most bacteria are neutral or beneficial to the host acting as symbiotic organisms (1). Stimulation of host cells by bacteria results in activation of the immune system that is critical for the elimination of pathogenic bacteria. Furthermore, recent studies of probiotics and resident microflora emphasize the importance of non-pathogenic bacteria to confer host resistance against various pathogens (1–3), elimination of neoplastic cells (1, 4), and prevention of allergic hyperresponsiveness (1, 5). These beneficial and non-beneficial interactions of the host with bacteria are mediated by recognition of bacterial components by host-specific receptor proteins (1).

The host immune system recognizes through specific receptor molecules that are commonly found in bacteria and therefore induces immune responses to a large number of microbes rather than to particular specific bacteria (6). Several membrane-bound Toll-like receptors (TLRs) and cytosolic Nod proteins are involved in host recognition of bacterial components, and regulate innate and acquired immune responses by activating transcription factors including NF-κB. These proteins include Nod1 and Nod2, which recognize particular structures in bacterial peptidoglycan (PGN)-related molecules (7), TLR2 and TLR4, which recognize membrane-bound molecules such as bacterial lipoproteins and lipopolysaccharide (LPS), respectively (8).

Nod1 is the founding member of a protein family of innate immune receptors that contain nucleotide-oligomerization domain (NOD) and ligand-recognizing leucine-rich repeats, and are comprised of more than 20 members including Nod2, Cryopyrin, and Ipaf (7, 9). Whereas Nod2 recognizes the muramyl dipeptide (MDP) structure in PGN-related molecules, Nod1 recognizes the essential iE-DAP dipeptide that is uniquely found in PGN of all Gram-negative bacteria and certain Gram-positive bacteria (10–13). Both iE-DAP and MDP structures are found in insoluble intact PGN, intermediates of PGN synthesis, and cleaved PGN products produced during bacterial growth and PGN recycling (15, 16). Several studies have shown that several small molecules containing the iE-DAP or MDP core structures, but not intact PGN, stimulate Nod1 and Nod2, respectively (10–13). Synthetic compounds containing the minimal Nod1 and Nod2 stimulatory structures induce and/or enhance NF-κB activation, chemokine and cytokine secretion, resistance against pathogens, antigen-specific antibody production, and delayed-type hypersensitivity reac-

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2 The abbreviations used are: TLR, Toll-like receptor; mDAP, meso-diaminopimelic acid; iE-DAP, γ-D-glutaminyl-γ-D-glutamylmeso-diaminopimelic acid; A-iE-DAP, L-alanyl-γ-D-glutaminylmeso-diaminopimelic acid; GlcNAc, N-acetylglicosaminyl; anhMurNAc, anhydro-N-acetylmuramyl; HEK293, human kidney embryo 293; WT, wild-type; PGN, peptidoglycan; LPS, lipopolysaccharide; NOD, nucleotide-oligomerization domain; MDP, muramyl dipeptide; GFP, green fluorescent protein; BHI, brain heart infusion; RICK, rip-like interacting clarp kinase.
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**TABLE 1**

| Bacteria strain | Gram classification | PGN type |
|-----------------|----------------------|----------|
| E. coli K12     | Negative             | mDAP     |
| Salmonella typhimurium SL133 | Negative         | mDAP     |
| L. pneumophila O21 | Negative            | mDAP     |
| Pseudomonas aeruginosa PA01 | Negative       | mDAP     |
| B. fragilis NCTC10581 | Negative         | mDAP     |
| B. vulgaris ATCC8482 | Negative       | mDAP     |
| P. putida N395*  | Negative             | mDAP     |
| B. subtilis natto N146* | Positive      | mDAP     |
| B. cereus N144*  | Positive             | mDAP     |
| B. megaterium N1409* | Positive       | mDAP     |
| B. pumilus N408*  | Positive             | mDAP     |
| L. monocytogenes EGD | Positive        | mDAP     |
| L. plantarum ATCC8041 | Positive     | mDAP     |
| L. pentosus ATCC11580 | Positive     | mDAP     |
| C. amylolactum N1355* | Positive        | mDAP     |
| S. aureus ATCC25923 | Positive          | l-Lys    |
| S. epidermidis N1359* | Positive       | l-Lys    |
| R. mucilaginosa N1343* | Positive       | l-Lys    |
| C. xerosis N1355*  | Positive             | Unknown  |

* These clones were isolated and identified as described under “Experimental Procedures.”

Ligand Compounds, Plasmids, Culture Cells, and Bacteria Strains—Cellosyl-treated PGN from *Bacillus subtilis*, synthetic compounds, iE-DAP, A-iE-DAP, MDP, sBLP (Pam3-Cys-OH), CpG, and poly(I:C) have been described (17). Escherichia coli O55:B5 LPS was purchased from Sigma. The LPS was free of contamination with Nod1- and Nod2-stimulatory activity as detected by the HEK293T bioassay as described (25).

pMX2-HA-Nod2, pcDNA3-MD2-FLAG, pcDNA3-TLR4-FLAG, and pEGFP/NF-κB have been described (10, 26), pCMV-SPORT6-hNod1 (cDNA clone MGC:17074) was obtained from Invitrogen. The plasmid pMSCV-puro-Nod1-FLAG was generated by subcloning the open reading frame of FLAG-tagged Nod1 from pcDNA3-Nod1-FLAG (10) into pMSSV-puro (Invitrogen). pBR322-amia-His was generated by subcloning the His-tagged open reading frame of amia amplified from *E. coli* K12 by PCR using amia-specific primers and subcloned into the SspI and PstI sites of pBR322.

Mouse macrophage RAW264.7, human embryonic kidney (HEK) 293T, and neuronal 293 cells were cultured in RPMI1640 and Dulbecco’s modified Eagle media, respectively, containing 10% heat-inactivated fetal calf serum with 100 units/ml penicillin and 100 μg/ml streptomycin (all culture reagents from Invitrogen) as described (10, 17). HEK293 constitutively expressing Nod1-FLAG and NF-κB-dependent GFP reporter plasmid (HEK1G) was generated by transfection of pMSCV-puro-Nod1-FLAG and NFκB-eGFP followed by antibiotic selection. Mouse macrophages (mFb) were derived from the bone marrow of 6-week-old B6 wild-type (WT) and Nod1-deficient mice as described (17). Mesothelial cells were prepared from B6 WT and RICK-deficient mice as described (27, 28). The mouse studies were approved by the University of Michigan Committee on Use and Care of Animals.

Bacteria used in Fig. 5 and their references are listed in Table 1. We obtained *Legionella pneumophila* O2 from Dr. R. R. Isberg (Tufts University), *Listeria monocytogenes* EGD from Dr. M. O’Riordan (University of Michigan), and *Bacteroides vulgatus* ATCC8482 and *Bacteroides fragilis* BCTC10581 (29) from Dr. T. Kirikae (Institute Medical Center of Japan), *E. coli* mutants MC1061, MHD45, MHD52, and MHD63 (30) from Dr. W. Vollmer (University of Tubingen, Germany), and TP71, TP73, TP72/pNU404 (31) from Dr. J. T. Park (Tufts University).

**Stability Assay of Nod1 Ligands**—For heat stability, synthetic ligands or bacterial fractions were heated at 98 °C for 30 min. For pH stability, synthetic Nod1 ligands were treated with 1 M HCl or NaOH for 12 h at 55 °C and neutralized with 10-fold excess volume of 1 M HEPES (pH 7.4). To determine the stability of bacterial molecules for host stimulation, synthetic ligands or Cellosyl-treated soluble PGN were incubated with HEK293T cells or bone marrow-mb. 24 h postincubation the Nod1-stimulatory activity in the medium containing the ligands was determined by the HEK293T bioassay as described (25).

**Isolation of Bacteria and Construction of Bacterioarray**—*Corynebacterium amycolatum* NI402, *Corynebacterium xerosis* NI355, *Staphylococcus epidermidis* NI379, and *Rothia mucilaginosa* NI343 were isolated from the body of a healthy donor. *P. putida* NI395 was isolated from a household. Soil bacteria for bacterioarray were isolated from the University of Michigan Arboretum. Soil samples were suspended in water and plated in L-broth and BHI agar. 1920 bacterial colonies were picked up randomly and aerobically cultured with L-broth or BHI medium in 96-well plates at 37 °C for 48 h. Bacterial cells and media were inactivated at 75 °C for 1 h. Reporter HEK1G cells were incubated with the medium containing bacterial cultures and media at 1:100 dilution. 24 h post-stimulation, the cells were suspended in reporter lysis buffer (Promega) and NF-κB-dependent transcription activity was determined by GFP inten-
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**FIGURE 1.** Ligand-specific NF-κB activation in human embryonic kidney cells expressing specific receptors. A, HEK293T cells were transfected with expression plasmids of Nod1, Nod2, TLR4/MD2, or vector control in the presence of pBxIV-luc and pEF1BOS-β-Gal. 8 h post-transfection, cells were treated with fresh medium containing 5 ng/ml iE-DAP, 2.5 ng/ml MDP, 20 ng/ml LPS, 1 μg/ml synthetic bacterial lipoproteins, 2 μg/ml CpG, 2.5 μg/ml poly(I:C) or left alone. 24 h post-transfection, NF-κB-dependent transcription activity was determined as described (4). The results shown are given as mean ± S.D. of triplicate cultures and are representative of three experiments. The level of NF-κB-dependent transcription activity in the presence of receptor-specific stimulation without ligands is given as 1. The absolute levels of NF-κB-dependent transcription activities for Nod1, Nod2, and TLR4 stimulation were 21.1-, 2.5-, and 6.5-fold, respectively. B, dose-dependent ligand response in HEK293T cells expressing each receptor. HEK293T cells were transfected with expression plasmids of Nod1, Nod2, and TLR4/MD2 as described in A. 8 h post-transfection, cells were treated with fresh medium containing the indicated amount of each receptor-specific ligand. 24 h post-transfection, NF-κB-dependent transcription activity was determined. C, coexistence of other Nod and TLR ligands did not interfere with the bioassay. HEK293T were transfected with expression plasmids of Nod1, Nod2, and TLR4/MD2. 8 h post-transfection, cells were treated with fresh medium containing the indicated receptor-specific ligands in the presence of iE-DAP (Nod1), MDP (Nod2), and TLR4 (LPS). 24 h post-transfection, NF-κB-dependent transcription activity was determined. The level of NF-κB-dependent transcription activity in the presence of receptor-specific stimulation without second ligands is given as 100%. The absolute levels of NF-κB-dependent transcription activities for Nod1, Nod2, and TLR4 stimulation were 3.2-, 12.7-, and 2.9-fold, respectively.

Bacteria were identified by DNA sequencing using primers corresponding to the consensus sequences of 16S ribosomal RNA genes. The 16S rDNA was amplified from single bacterial colonies by 35 cycles of PCR at 94 °C for 1 min, 45 °C for 1 min, and 72 °C for 1 min, using the broad-range bacterial primers CCAAACTCTACGGGAGGCAGCAG and CATGGAC- AGGCAGGCAGCAG and 72 °C for 1 min, using the broad-range bacterial primers CCAAACTCTACGGGAGGCAGCAG and CATGGACAGGCAGCAG and 72 °C for 1 min, using the broad-range bacterial primers CCAAACTCTACGGGAGGCAGCAG and CATGGACAGGCAGCAG and 72 °C for 1 min, using the broad-range bacterial primers CCAAACTCTACGGGAGGCAGCAG and CATGGACAGGCAGCAG and 72 °C for 1 min, using the broad-range bacterial primers CCAAACTCTACGGGAGGCAGCAG and CATGGACAGGCAGCAG. The resulting sequences were subjected to an online BLASTN analysis (NCBI, National Institutes of Health), and sequenced from both ends using the fluoroquinolone Nod primers. HEK293T cells were transfected with expression plasmids of Nod1, Nod2, TLR4/MD2, or vector control in the presence of pBxIV-luc and pEF1BOS-β-Gal. 8 h post-transfection, cells were treated with fresh medium containing the indicated receptor-specific ligands in the presence of iE-DAP (Nod1), MDP (Nod2), and TLR4 (LPS). 24 h post-transfection, NF-κB-dependent transcription activity was determined. The bioassay was performed by serial dilution of lysate followed by centrifugation and filtration as described above for ligand fractions of bacterial cultures.

**HEK293T Bioassay for Specific Pathogen Receptors**—Ligand-dependent NF-κB activation was determined using a reporter assay. HEK293T cells transfected with expression plasmids of Nod1 (0.17 ng of pCMV-SPORT6-Nod1), Nod2 (33 ng of pMX2-HA-Nod2), or TLR4 (1.7 ng of pCDNA3-TLR4-FLAG) and MD2 (1.7 ng of pCDNA3-MD2-FLAG) in the presence of reporter plasmids, NF-κB-dependent pBxIV-luc, and control pEF1BOS-β-Gal as described (10). Briefly, HEK293T cells were transfected with expression plasmids by the calcium phosphate method and 8 h post-transfection cells were treated with fresh medium containing various ligands or bacterial products. 24 h post-transfection, ligand-dependent NF-κB activation was determined with reporter assay.

**RESULTS AND DISCUSSION**

Ligand-specific NF-κB Activation in Human Embryonic Kidney Cells Expressing Nod1, Nod2, or TLR4—Host cells recognize bacteria through several receptors that are specific for particular bacterial components (6). These receptors including Nod1, Nod2, and TLRs mediate innate and acquired immune responses against bacteria. However, the levels of microbial ligands present in bacteria that are capable of stimulating these...
receptors are poorly characterized. Because bacteria express multiple immunostimulatory molecules that synergistically activate NF-κB (10, 17, 32), determination of the ability of bacteria to stimulate receptor-specific activity using host cells that express multiple receptors (e.g. macrophages and DCs) is not feasible. Unlike macrophages, HEK293T cells express no or low levels of nod and TLR proteins and are insensitive to less than 100 ng/ml of synthetic microbial compounds capable of stimulating nod1, nod2, TLR2, TLR3, TLR4, and TLR9 (Fig. 1A). To determine the levels of receptor-specific immunostimulatory activity in bacterial cells and cultured media, HEK293T cells expressing nod1, nod2, and TLR4 were stimulated with their corresponding bacterial activators. The synthetic bacterial compounds that are specifically recognized by nod1, nod2, and TLR4, activated NF-κB in HEK293T cells expressing each specific receptor, but not parental HEK293T cells, in a dose-dependent manner (Fig. 1, A and B). Co-administration of two compounds that are recognized by distinct receptors did not induce significant synergistic effects (Fig. 1C). Therefore, the level of immunostimulatory activity for each host receptor could be determined by the HEK293T bioassay, even when bacteria and bacterial extracts contain different types of immunostimulatory molecules.

**Intracellular Localization of Bacteria Is Not Required for Nod1 Stimulation**—Previous studies suggested that intracellular localization of bacterial components is important for the interaction between host cells and bacteria through nod1 and nod2 (11, 17, 25, 33). However, there is also evidence that extracellular MDP induces immune responses through nod2 (34, 35), presumably through incorporation of MDP into cells through specific transporter systems (36). Consistent with the latter, we found that extracellular administration of iE-DAP and A-iE-DAP, two soluble synthetic nod1 ligands, induces NF-κB activation in HEK293 cells expressing nod1 in the absence of reagents that mediate their intracellular uptake (Fig. 2A). Significantly, stimulation of cells with nod1-stimulatory molecules in the presence of calcium phosphate particles that facilitate their intracellular internalization increased 3–5-fold the levels of NF-κB activation (Fig. 2B). These results suggest that extracellular administration of nod1 ligand molecules is sufficient to stimulate nod1, but enforced intracellular localization facilitates their ability to stimulate nod1.

To test if intracellular localization of bacteria is required to stimulate nod1, we first determined if MCP-1 secretion from mouse...
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Bone marrow-derived macrophages required intracellular localization of L. monocytogenes, a Gram-positive bacterium that contains mDAP-type PGN (37). Infection of macrophages with L. monocytogenes induced MCP-1 secretion (Fig. 2B). Significantly, infection with a Listeria hly mutant strain deficient in listeriolysin O (LLO), a factor required for the escape of the bacterium from the vacuole into the host cytosol, induced significant, although reduced levels of MCP-1 secretion when compared with WT bacteria (Fig. 2B). Thus, cytosolic localization of L. monocytogenes is not essential for MCP-1 secretion. Both WT and Δhly strains induced MCP-1 secretion from Nod1-deficient macrophages, suggesting that MCP-1 secretion elicited by L. monocytogenes is Nod1-independent (Fig. 2B).

Because Nod1-independent MCP-1 secretion from macrophages is probably due to stimulation through multiple host receptors in macrophages, we infected HEK293T cells expressing Nod1 with these strains of L. monocytogenes. As expected, unlike the WT bacterium, the Δhly mutant did not translocate into the cytosol of HEK293T cells (Fig. 2C). Notably, both WT and Δhly mutant L. monocytogenes induced similar levels of Nod1- and Nod2-dependent NF-κB activation but, as expected, they failed to induce TLR4-dependent activation in TLR4-negative HEK293T cells (Fig. 2D). These results suggest that extracellular Nod1- and Nod2-stimulatory molecules present in L. monocytogenes are important for the stimulation of Nod1 and Nod2.

Immunostimulatory Activity in Various Bacterial Environments—Previous studies demonstrated that Nod1-stimulatory activity associated with bacteria is highly stable in acidic (1 M HCl), alkaline (1 M NaOH), heated (100 °C), and phenol (10). To verify this, iE-DAP, a synthetic Nod1 ligand, was treated with 1 M HCl, which is more acidic than gastric fluid, and 1 M NaOH or boiled at 100 °C. Whereas control ligand compounds, MDP and LPS, ligands of Nod2 and TLR4, respectively, were inactivated by treatment with HCl and NaOH, the Nod1-stimulatory activity of iE-DAP was not affected by these treatments (Fig. 3A). Furthermore, incubation of iE-DAP or PGN with HEK293T or macrophages did not affect their ability to stimulate Nod1 (Fig. 3B), consistent with the fact that no host cellular enzymes are known to cleave and inactivate iE-DAP. These results indicated that Nod1-stimulatory activity in bacteria is more stable than Nod2- and TLR4-stimulatory molecules. Therefore, it is possible that materials and environments such as food and soil might contain a significant ability to induce Nod1-dependent immune responses even when they were once contaminated but presently free of bacteria. To test this, we assessed the ability of water-soluble extracts from soil, fermented foods, and mouse bedding materials to stimulate Nod1 and Nod2. Notably, all samples from soil, fermented foods, mouse contents of ileum and cecum, and mouse bedding materials before and after animal housing significantly contained Nod1- and/or Nod2-stimulatory activity (Fig. 3C). The highest Nod1-stimulatory activity (40 ± 13 kU/ml) and Nod2-stimulatory activity (0.84 ± 0.21 kU/ml) were found in Natto, a traditional Japanese food product derived from fermented soybeans. In contrast, water-soluble extracts from several yogurt products failed to stimulate Nod1 but stimulated Nod2 (Fig. 3C). These results suggest that bacterial products in foods and bacterial environments around human and mice possess the ability to stimulate the hosts immune systems through Nod1 and/or Nod2 even in the absence of living bacteria.

Screening of Bacteria for Nod1-stimulatory Activity—Previous studies suggest that the levels of Nod1-stimulatory activity in bacterial cellular extracts are different among bacterial species (11). However, these early studies are limited in that they were based on a few selected bacteria. Therefore, we prepared a panel of ~2,000 randomly isolated bacteria from soil samples by culture on L-broth and BHI medium plates. The clones of

FIGURE 4. Screening of bacteria for Nod1-stimulatory activity. A, HEK293 cells constitutively expressing Nod1 with NF-κB-dependent GFP reporter (HEK1G) were stimulated with the indicated amount of iE-DAP, heat-inactivated culture of the bacterial clone B. simplex L2-H5, or left alone (−) as control. 24 h post-stimulation, green fluorescence signals were detected in the cells with excitation at 450–490 nm. B, bacterial cells were isolated from soil and cultured in L-broth (LB) and BHI media. HEK1G cells were incubated with the heat-inactivated bacterial cultures at 1:100 volumes. 24 h post-stimulation, NF-κB-dependent GFP reporter (HEK1G) were stimulated with the indicated amount of iE-DAP, heat-inactivated culture of the bacterial clone B. simplex L2-H5, or left alone (−) as control. 24 h post-stimulation, NF-κB-dependent transcription activity was determined. The level of NF-κB activation by each bacterial clone is shown as intensity of GFP color.
the library referred here as bacterioarray were arrayed in 96-well plates and screened for their ability to induce Nod1-dependent NF-κB activation. HEK293 cells carrying Nod1 and a NF-κB-dependent GFP reporter gene (HEK1G) were stimulated with heat-inactivated bacteria and the ability to induce Nod1-dependent NF-κB activation was estimated by the level of fluorescence intensity in each stimulated HEK1G reporter cell (Fig. 4). The bacteria with high Nod1-stimulatory activity induced strong expression of GFP when compared with that induced by control synthetic Nod1-stimulatory compounds (Fig. 4A). This suggests that extracellular administration of natural bacterial products is as Nod1 stimulatory as that of synthetic ligand compounds. Incubation of HEK1G reporter cells with about 2,000 independently derived bacterial clones resulted in different levels of Nod1 stimulation (Fig. 4B). The 48 bacterial clones that possessed the highest Nod1-stimulatory activity were identified and characterized by colony-forming assay on culture plates, morphological assay, and 16 S rRNA sequencing. All bacteria with the strongest Nod1-stimulatory activity were found to belong to the genus Bacillus. They included 29 clones for Bacillus simplex, 16 for Bacillus pumilus, 1 for Bacillus cereus, 1 for Bacillus fusiformis, and 1 for a novel Bacillus strain (Table 2). These results indicate that the major soil bacteria that are recognized by host cells though Nod1 are Bacillus species.

**Diversity of Nod1- and Nod2-stimulatory Activity among Bacterial Species**—Several iE-DAP- and MDP-containing molecules are known to be released from bacterial cells during bacterial growth (30, 31). Therefore, it is possible that PGN-derived molecules released from bacterial cells into the culture media could stimulate Nod1 and Nod2. To test this hypothesis, we prepared medium supernatants from overnight culture of several bacteria and the level of Nod1- and Nod2-stimulatory activity in the supernatants was determined by the HEK293

### Table 2: Identification of Nod1-stimulatory bacteria isolated from soil

| Clone ID No. | Identity | GenBank No. | Identity | PGN type | Gram classification | ΔF |
|--------------|----------|-------------|----------|----------|---------------------|----|
| B2-C4        | B. simplex | AY833099   | 100      | mDAP-type | Gram-positive       | 11.7 |
| L2-E5        | B. simplex | AY833099   | 100      | mDAP-type | Gram-positive       | 11.6 |
| L2-C7        | Bacillus sp. | A920000    | 99       | mDAP-type | Gram-positive       | 11.4 |
| B3-F6        | B. simplex | AY833099   | 100      | mDAP-type | Gram-positive       | 11.2 |
| B3-F7        | B. simplex | AY833099   | 100      | mDAP-type | Gram-positive       | 11.1 |
| L3-G6        | B. simplex | AY833099   | 100      | mDAP-type | Gram-positive       | 11   |
| L3-C8        | B. simplex | AY833099   | 100      | mDAP-type | Gram-positive       | 10.8 |
| L3-F1        | B. simplex | AY833099   | 100      | mDAP-type | Gram-positive       | 10.5 |
| B3-E4        | B. simplex | AY833099   | 100      | mDAP-type | Gram-positive       | 10.4 |
| L3-C7        | B. simplex | AY833099   | 100      | mDAP-type | Gram-positive       | 10.4 |
| L3-B7        | B. simplex | AY833099   | 100      | mDAP-type | Gram-positive       | 10.3 |
| L3-B10       | B. simplex | AY833099   | 100      | mDAP-type | Gram-positive       | 10.3 |
| L3-F5        | B. cereus | DQ234855   | 100      | mDAP-type | Gram-positive       | 10.3 |
| L5-A7        | B. simplex | AY833099   | 100      | mDAP-type | Gram-positive       | 9.29 |
| L5-A4        | B. simplex | AY833099   | 100      | mDAP-type | Gram-positive       | 7.1 |
| B7-F1        | B. pumilus | AF526907   | 100      | mDAP-type | Gram-positive       | 6.77 |
| B7-H4        | B. pumilus | AF526907   | 100      | mDAP-type | Gram-positive       | 6.76 |
| B7-D4        | B. pumilus | AF526907   | 100      | mDAP-type | Gram-positive       | 6.46 |
| L8-E4        | B. simplex | AY833099   | 100      | mDAP-type | Gram-positive       | 6.43 |
| B7-E1        | B. pumilus | AY462205   | 100      | mDAP-type | Gram-positive       | 6.34 |
| B8-G2        | B. pumilus | AF526907   | 100      | mDAP-type | Gram-positive       | 6.28 |
| L5-A5        | B. simplex | AY833099   | 100      | mDAP-type | Gram-positive       | 6.26 |
| L8-A9        | B. simplex | AY833099   | 100      | mDAP-type | Gram-positive       | 6.24 |
| B10-D9       | B. pumilus | AF526907   | 100      | mDAP-type | Gram-positive       | 6.06 |
| L5-E8        | B. simplex | AY833099   | 100      | mDAP-type | Gram-positive       | 6.03 |
| L5-D8        | B. simplex | AY833099   | 100      | mDAP-type | Gram-positive       | 5.89 |
| L4-E4        | B. simplex | AY833099   | 100      | mDAP-type | Gram-positive       | 5.85 |
| B10-D4       | B. pumilus | AF526907   | 100      | mDAP-type | Gram-positive       | 5.82 |
| B5-A7        | B. pumilus | AF526907   | 100      | mDAP-type | Gram-positive       | 5.81 |
| L5-A9        | B. simplex | AY833099   | 100      | mDAP-type | Gram-positive       | 5.77 |
| L10-G7       | B. pumilus | AY462205   | 100      | mDAP-type | Gram-positive       | 5.76 |
| B9-C7        | B. pumilus | AF526907   | 100      | mDAP-type | Gram-positive       | 5.73 |
| B5-H5        | B. simplex | AY833099   | 100      | mDAP-type | Gram-positive       | 5.69 |
| B7-F4        | B. pumilus | AF526907   | 100      | mDAP-type | Gram-positive       | 5.63 |
| B6-A7        | B. pumilus | AF526907   | 100      | mDAP-type | Gram-positive       | 5.54 |
| L4-F6        | B. simplex | AY833099   | 100      | mDAP-type | Gram-positive       | 5.51 |
| L6-F8        | B. simplex | AY833099   | 100      | mDAP-type | Gram-positive       | 5.45 |
| B9-G4        | B. pumilus | AF526907   | 100      | mDAP-type | Gram-positive       | 5.45 |
| B8-H3        | B. pumilus | AF526907   | 100      | mDAP-type | Gram-positive       | 5.45 |
| B8-B5        | B. pumilus | AF526907   | 100      | mDAP-type | Gram-positive       | 5.44 |
| L5-B7        | B. simplex | AY833099   | 100      | mDAP-type | Gram-positive       | 5.41 |
| L7-A9        | B. simplex | AY833099   | 100      | mDAP-type | Gram-positive       | 5.27 |
| L4-D8        | B. simplex | AY833099   | 100      | mDAP-type | Gram-positive       | 5.16 |
| L6-G5        | B. simplex | AY833099   | 100      | mDAP-type | Gram-positive       | 5.15 |
| L4-D12       | B. simplex | AY833099   | 100      | mDAP-type | Gram-positive       | 5.14 |
| B5-B5        | B. simplex | AY833099   | 100      | mDAP-type | Gram-positive       | 5.06 |
| L5-G6        | B. fusiformis | AB167231 | 100      | mDAP-type | Gram-positive       | 5.03 |

Bacteria were isolated from soil and identified as described under “Experimental Procedures.” The bacteria that possess the highest Nod1 stimulatory activity were screened by bacterioarray analysis shown in Fig. 4. Bacteria are shown with identification number (ID No.) and the level of fluorescence intensity (ΔF). The ΔF is given as a comparison with that of non-stimulated HEK1G cells. The identity of 48 bacteria that possess the highest Nod1 stimulatory activity in bacterioarray was determined by 16 S rDNA sequencing. The nucleotide sequence with highest homology to each clone is given as GenBank accession number and the percentage of the identity. The PGN types and Gram-staining classification of the bacteria are given according to Ref. 35.
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FIGURE 5. Nod1-, Nod2-, and TLR4-stimulatory activity in various bacterial cells and culture media. Bacterial cells (cell) and culture supernatants (sup) were prepared from overnight cultures of bacteria listed in Table 1. For B. simplex, new isolated clone L2-H5 was used. The bacterial numbers per ml were $3.2 \times 10^9$, $3.5 \times 10^9$, $4.2 \times 10^9$, $5.5 \times 10^9$, $6.7 \times 10^9$, $8.9 \times 10^9$, $2.0 \times 10^9$, $2.5 \times 10^9$, $6.21 \times 10^9$, $2.0 \times 10^9$, $1.2 \times 10^9$, $1.5 \times 10^9$, $3.5 \times 10^9$, $3.3 \times 10^9$, $6.1 \times 10^9$, $5.2 \times 10^9$, $5.4 \times 10^9$, and $5.4 \times 10^9$, respectively. The Nod1-, Nod2-, and TLR4-stimulatory activities were determined by the HEK293T bioassay as described under “Experimental Procedures” and given with kilounits/ml. The activity of bacterial cell extract was given with kilounits/ml of the original culture volume. One unit (U) of the Nod1-, Nod2-, and TLR4-stimulatory activity is equivalent to those of 1 ng of synthetic iE-DAP, MDP, and purified E. coli O55:B5 LPS, respectively.

FIGURE 6. MCP-1 secretion induced by Bacillus species required RICK in mesothelial cells. Mesothelial cells were prepared from WT and RICK-deficient mice as described under “Experimental Procedures.” $2 \times 10^6$ cells were incubated with 10-fold diluted medium supernatants from overnight cultures of B. cereus (Bc), B. megaterium (Bm), S. aureus (Sa), E. coli (Ec), or left alone. As controls, cells were incubated with 5 μg/ml synthetic Nod1 ligand KF1B, 50 ng/ml E. coli LPS, poly(I:C), and 10 ng/ml tumor necrosis factor-α. 24 h post-stimulation, the levels of MCP-1 secretion was determined by enzyme-linked immunosorbent assay.

Bioassay. Both Nod1- and Nod2-stimulatory activity was detected in the supernatants (Fig. 5), suggesting that the Nod1 and Nod2 ligands are released from bacteria. As expected from the presence of the conserved MDP structure in PGN (37), the medium supernatant and cell extract of B. subtilis contained 10–30-fold higher Nod2-stimulatory activity than S. aureus (Fig. 5). Similarly, B. subtilis, E. coli, and Lactobacillus plantarum possess the essential iE-DAP structure, but they express vastly different levels of Nod1-stimulatory activity (Fig. 5). These results indicate that the levels of intracellular and released Nod1- and Nod2-stimulatory activity vary greatly in different bacterial species. We also found that the higher Nod1-stimulatory activity was associated with the culture supernatants, whereas higher Nod2-stimulatory activity with associated extracts from whole cells of almost all bacteria (Fig. 5). As a control, TLR4-stimulatory activity was primarily found associated with whole cell extracts of Gram-positive bacteria such as E. coli and S. typhimurium, but not from Gram-positive bacteria that lack LPS (Fig. 5).

The results using the 293T bioassay suggest that the Bacillus species possess high Nod1- and Nod2-stimulatory activity. To test if the immune response to Nod1/Nod2-stimulatory bacteria is dependent on Nod1 and Nod2 signaling, we determined the levels of MCP-1 secreted in response to bacteria by mesothelial cells from WT mice and mice lacking RICK, a common downstream mediator of both Nod1 and Nod2 signaling, we determined the levels of MCP-1 secreted in response to bacteria by mesothelial cells from WT mice and mice lacking RICK, a common downstream mediator of both Nod1 and Nod2 signaling. We found that the levels of MCP-1 secreted in response to bacteria by mesothelial cells from WT mice and mice lacking RICK were reduced, indicating that RICK is required for MCP-1 secretion from Bacillus-stimulated mesothelial cells. Compared with those from the Bacillus species, culture supernatant from E. coli possessed the ability to induce MCP-1 secretion in RICK-deficient cells, probably due to the presence of...
In a previous study, we found that Nod1 stimulation induces RICK-independent immunostimulatory molecules including LPS. Therefore, this result suggests that Nod signaling is important for recognition of the Bacillus species but not Gram-negative bacterium E. coli by mesothelial cells.

amiA and ampD Are Not Essential for Release of Nod1-stimulatory Activity—To characterize mechanisms by which Nod1- and Nod2-stimulatory molecules are released to the culture medium from bacterial cells, we tested if mutations of genes that regulate PGN turnover in E. coli affect the levels of Nod1- and Nod2-stimulatory activity in the culture supernatants. These genes include sltY, which encodes lytic glycosyltransferase to produce GlcNAc-anhMurNAc-oligopeptides (16, 30), ampD, amiA, and related ami genes, that encode N-acetylmuramyl-L-alanine amidases to produce tri- and tetrapeptides containing iE-DAP structures (30, 31). The analysis of bacterial cell extracts and culture supernatants from E. coli mutant strains that lack sltY, ampD, amiA, and amiA homologues (amiB and amiC) revealed that mutations in these genes did not abolish Nod1-stimulatory activity in the culture supernatants (Fig. 7). This is not due to redundancy of immunostimulation from other bacterial components because HEK293T cells lack the ability to respond to Nod- and TLR-stimulatory molecules (10–14, 17, 25, also see Fig. 1). Indeed, amiB and ampD/E deletions resulted in a slight increase in Nod1-stimulatory activity in the bacterial cell extract and cultured medium (Fig. 7). The increased Nod1-stimulatory activity in AmpD was reversed by introduction of exogenous AmpD (Fig. 7C). This is consistent with previous reports that showed that GlcNAc-anhMurNAc-oligopeptides are Nod1 ligands (10, 14). However, the sltY mutation did not decrease the Nod1-stimulatory activity (Fig. 7A, compare MHD52 and MHD63), suggesting that the major Nod1-stimulatory molecules released from ampD+ strains are not GlcNAc-anhMurNAc-oligopeptides. Therefore, Nod1-stimulatory molecules released from E. coli to the culture supernatant are mediated by a novel mechanism.

A previous study, we found that Nod1 stimulation induces local recruitment of neutrophils, a process that is known to be critical for bacterial clearance (17). Here we identified several Bacillus species as the bacteria that possess the highest Nod1-stimulatory activity after screening randomly isolated bacteria from soil and comparison with that found in a panel of non-pathogenic and pathogenic bacteria. In earlier studies, we found that iE-DAP is a core structure of Nod1-stimulatory activity in mDAP-containing PGN from B. subtilis, a Gram-positive bacterium (10, 14). However, another group suggested that heated extracts from certain strains of B. subtilis contain lesser Nod1-stimulatory activity than several Gram-negative bacteria (11, 14). Here we provide further evidence that all tested strains of B. subtilis possess significant Nod1-stimulatory activity. Indeed, the quantitative bioassay demonstrated that Nod1-stimulatory activity from B. subtilis is higher than that found in many Gram-negative bacteria. The products derived from insoluble B. subtilis PGN digested with muramidase in vitro contain amidated mDAP that possesses 10-fold lesser Nod1-stimulatory activity than non-amidated PGN products (14). It is possible that the natural Nod1-stimulatory molecules and those derived after treatment of insoluble PGN with muramidase are different. Alternatively, the higher amounts of natural Nod1-stimulatory molecules in bacterial cells and culture supernatants might compensate their relatively lower ability to stimulate Nod1. Indeed, preliminary analysis suggests that the culture supernatant from B. subtilis has a higher amount of ninhydrin-positive mDAP-containing molecules than that from E. coli as detected in the TLC assay. Because all tested Bacillus species were found to possess the highest Nod1-stimulatory activity, the potent activity appears to be a general feature of the genus Bacillus. The genus Bacillus includes many non-pathogenic and several pathogenic bacteria including B. anthracis, some strains of B. cereus, Bacillus mycoides, Bacillus thuringiensis, and B. subtilis (39). Therefore, Nod1 may play important roles in the clearance of these pathogenic bacilli, a hypothesis that is being tested in our laboratory.

Bacillus species are the most common bacteria in human and animal environments and many of them are non-pathogenic. Stimulation of intestinal epithelial cells with B. subtilis induces secretion of several cytokines and chemokines, but not tumor necrosis factor-α (40), which is similar to that found with Nod1 ligands (17). Therefore, recognition of Bacillus products by Nod1 may have a different role in host immunity than that traditionally associated with acute immune responses against pathogenic bacteria. For example, the Th1/Th2-oriented immune homeostasis might be modified by the interaction of the host with bacteria through Nod1. Environmental factors that possess bacteria and bacterial products are known to affect the susceptibility to human diseases including Th2-oriented allergic diseases and autoinflammatory diseases (41). Genetic variation in the Nod1 locus is associated with increased susceptibility to allergic diseases (23, 24). Therefore, recognition of bacteria through the interaction of host

FIGURE 7. Amidases are not essential for production of Nod1-stimulatory molecules in E. coli. A, the Nod1- and Nod2-stimulatory activities in bacterial cell extracts (cell) and culture supernatants (sup) from WT E. coli MC106, mutant MHD45 (amiAΔ, amiCΔ), MHD52 (amiAΔ, amiBΔ, amiCΔ), and MHD63 (amiAΔ, amiBΔ, amiCΔ, sltYΔ) were determined by the HEK293T bioassay. B, the Nod1- and Nod2-stimulatory activities in bacterial materials from E. coli DH5α carrying AmiA expression plasmid and vector control (−) were determined by the HEK293T bioassay. C, the Nod1- and Nod2-stimulatory activities in bacterial materials from E. coli strains, WT TP71, TP73 (ampDΔ, ampEΔ), and TP73 carrying AmpD expression plasmid (TP73/D) were determined by the HEK293T bioassay.
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Nod1 with Nod1 ligands present at environmental sites might contribute to the homeostatic regulation of the immune system. Consistent with the latter, immunostimulation of mice with FK-565, a synthetic Nod1 ligand, results in broad resistance against a wide range of pathogens and elimination of cancer cells (15). Further studies are required to understand the role of nonpathogenic Bacillus species in the homeostasis of the immune system.

A surprising finding of the current studies was the high levels of Nod1-stimulatory activity in culture supernatant as compared with that found in bacterial cell extracts. The Nod1-stimulatory activity from bacteria, unlike Nod2- and TLR4-stimulatory activities, is highly stable and cannot be removed by boiling and by treatment at extreme pH conditions. These findings suggest that Nod1-stimulatory molecules in environmental sites and foods might often stimulate the hosts immune system through Nod1, even after pasteurization or sterilization. Although free stem peptides from PGN are known to stimulate Nod1 (14), genetic deficiency of enzymes producing these stem peptides did not abolish the Nod1-stimulatory activity in bacterial cell extracts and cultured medium. Structural determination of natural Nod1 ligand molecules from various bacterial environments will facilitate our understanding on the interaction between hosts and bacteria.

In this study, we identified bacteria possessing the highest Nod1-stimulatory activity after screening of bacteria randomly isolated from soil. This strategy is useful to identify bacteria that stimulate the host immune system through other specific pathogen receptors such as TLRs. The amount of LPS, the TLR4 ligand, can be estimated by the Limulus aggregation assay (42, 43). However, the Limulus assay is based on the reactivity of Limulus agglutinin with LPS but does not assess TLR4-stimulatory activity (44, 45). Moreover, bacteria may contain multiple molecules that inhibit the hosts recognition though TLR4. Indeed, we found that LPS-containing Gram-negative bacteria P. aeruginosa and P. putida has a very weak ability to stimulate TLR4, consistent with the fact that Pseudomonas aeruginosa LPS contain low TLR4-stimulatory activity (46, 47). Moreover, TLR4-stimulatory activity of individual bacteria is expected to be affected not only by the relative bioactivity but also by the amount of LPS, inhibitors of TLR4 signaling, and other molecules that physically and chemically may affect the interaction between LPS and TLR4. Therefore, randomly isolated bacteria libraries, here termed bacterioarrays, is a novel strategy to investigate in a comprehensive manner the interaction between host cells and bacterial populations. The comprehensiveness of the bacterioarray is similar to that of gene microarrays and expression-tag sequence libraries used in the analysis of gene expression. Construction of bacterioarrays from bacteria present at various environmental sites might provide novel insights into the relationship between host immunostimulation and the environmental factors that influence human diseases.

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REFERENCES

1. Rastall, R. A. (2004) J. Nutr. 134, 2022S–2026S
2. Guarnier, F., and Malagelada, J. R. (2003) Lancet 361, 512–519
3. Mack, D. R., and Lebel, S. (2004) Curr. Opin. Gastroenterol. 20, 22–26
4. Lin, J., Lin, E., and Nemunaitis, J. (2004) Curr. Opin. Mol. Ther. 6, 629–639
5. Tsujillo, C., and Erb, K. J. (2003) Int. J. Med. Microbiol. 293, 123–131
6. Janeway, C. A., Jr., and Medzhitov, R. (2002) Annu. Rev. Immunol. 20, 197–216
7. Inohara, N., Chamaillard, M., McDonald, C., and Nuñez, G. (2004) Annu. Rev. Biochem. 74, 355–383
8. Akira, S., and Takeda, K. (2004) Nat. Rev. Immunol. 4, 499–511
9. Inohara, N., Koseki, T., del Peso, L., Hu, Y., Yee, C., Chen, S., Carrio, R., Merino, J., Liu, D., Ni, J., and Nuñez, G. (1999) J. Biol. Chem. 274, 14560–14567
10. Chamaillard, M., Hashimoto, M., Horie, Y., Masumoto, J., Qiu, S., Saab, L., Ogura, Y., Kawasaki, A., Fukase, K., Kusumoto, S., Valvano, M. A., Foster, S. J., Mak, T. W., Nuñez, G., and Inohara, N. (2003) Nat. Immunol. 4, 702–707
11. Girardin, S. E., Boneca, I. G., Carneiro, L. A., Antignac, A., Jéhanno, M., Viala, J., Tedin, K., Taha, M.-K., Labigne, A., Zaehringer, U., Coyl, A. J., Di Stefano, P. S., Bertin, J., Sansonetti, P. J., and Philpott, D. J. (2003) Science 300, 1584–1587
12. Inohara, N., Ogura, Y., Fontalba, A., Gutierrez, O., Pons, F., Crespo, J., Fukase, K., Inamura, S., Kusumoto, S., Hashimoto, M., Foster, S. J., Moran, A. P., Fernandez-Luna, J. L., and Nuñez, G. (2003) J. Biol. Chem. 278, 5509–5512
13. Girardin, S. E., Boneca, I. G., Viala, J., Chamaillard, M., Labigne, A., Thomas, G., Philpott, D. J., and Sansonetti, P. J. (2003) J. Biol. Chem. 278, 8869–8872
14. Girardin, S. E., Travassos, L. H., Herve, M., Blanot, D., Boneca, I. G., Philpott, D. J., Sansonetti, P. J., and Mengin-Lecreux, D. (2003) J. Biol. Chem. 278, 41702–41708
15. Goto, T., and Aoki, H. (1987) Immunostimulants: Now and Tomorrow, pp. 99–108, Japan Scientific Societies Press, Tokyo
16. Hölzle, J. V. (1998) Microbiol. Mol. Biol. Rev. 62, 181–203
17. Masumoto, J., Yang, K., Varambally, S., Hasegawa, M., Tomlins, S. A., Qiu, S., Fujimoto, Y., Kawasaki, A., Foster, S. J., Horie, Y., Mak, T. W., Nuñez, G., Chinnaian, A. M., Fukase, K., and Inohara, N. (2006) Exp. Med. 203, 203–213
18. Hugot, J. P., Chamaillard, M., Zouali, H., Lesage, S., Cezard, J. P., Belaïche, J., Almer, S., Taha, M.-K., Labigne, A., Zaehringer, U., Coyl, A. J., Di Stefano, P. S., Bertin, J., Sansonetti, P. J., and Mengin-Lecreux, D. (2003) J. Biol. Chem. 278, 41702–41708
19. Ogura, Y., Boneca, I. G., Inohara, N., Ogura, Y., Fontalba, A., Gutierrez, O., Pons, F., Crespo, J., Fukase, K., Inamura, S., Kusumoto, S., Hashimoto, M., Foster, S. J., Moran, A. P., Fernandez-Luna, J. L., and Nuñez, G. (2003) J. Biol. Chem. 278, 5509–5512
20. McGovern, D. P., Hysi, P., Ahmad, T., van Heel, D. A., Moffat, M. F., Carey, A., Cookson, W. O., and Jewell, D. P. (2005) Hum. Mol. Genet. 14, 1245–1250
21. Kabesch, M., Peters, W., Carr, D., Leupold, W., Weiland, S. K., and von Mutius, E. (2003) J. Allergy Clin. Immunol. 111, 813–817
22. Weidinger, S., Klopff, N., Rummel, L., Wagenpfel, S., Baurecht, H. J., Gauger, A., Darsow, U., Jakob, T., Novak, N., Schafer, T., Heinrich, J., Behrendt, H., Wichmann, H. E., Ring, J., and Illig, T. (2005) Clin. Exp. Allergy 35, 866–872
23. Hysi, P., Kabesch, M., Moffatt, M. F., Schedel, M., Carr, D., Zhang, Y., Boardman, B., von Mutius, E., Weiland, S. K., Leupold, W., Fritzsche, C., Klopff, N., Musk, A. W., James, A., Nuñez, G., Inohara, N., and Cookson, W. O. (2005) Hum. Mol. Genet. 14, 935–941
24. Weidinger, S., Klopff, N., Rummel, L., Wagenpfel, S., Novak, N., Baurecht, H. J., Groer, W., Darsow, U., Heinrich, J., Gauger, A., Schafer, T., Jakob, T., Behrendt, H., Wichmann, H. E., Ring, J., and Illig, T. (2005) J. Allergy Clin. Immunol. 116, 177–184
25. Inohara, N., Ogura, Y., Chen, F. F., Muto, A., and Nuñez, G. (2001) J. Biol. Chem. 276, 2551–2554
26. Wang, D., You, Y., Case, S. M., McAllister-Lucas, L. M., Wang, L., DiStefano, P. S., Nuñez, G., Bertin, J., and Lin, X. (2002) *Nat. Immunol.* 3, 830–835
27. Foley-Comer, A. J., Herrick, S. E., Al-Mishlab, T., Prele, C. M., Laurent, G. J., and Mutsaers, S. E. (2002) *J. Cell Sci.* 115, 1383–1389
28. Kobayashi, K., Inohara, N., Hernandez, L. D., Galan, J. E., Nuñez, G., Jane-way, C. A., Medzhitov, R., and Flavell, R. A. (2002) *Nature* 416, 194–199
29. Hashimoto, M., Kirikae, F., Dohi, T., Adachi, S., Kusumoto, S., Suda, Y., Fujita, T., Naoki, H., and Kirikae, T. (2002) *Eur. J. Biochem.* 269, 3715–3721
30. Heidrich, C., Templin, M. F., Ursinus, A., Merdanovic, M., Berger, J., Schwarz, H., de Pedro, M. A., and Höltje, J. V. (2001) *Mol. Microbiol.* 41, 167–178
31. Jacobs, C., Huang, L. J., Bartowsky, E., Normark, S., and Park, J. T. (1994) *EMBO J.* 13, 4684–4694
32. Tada, H., Aiba, S., Shibata, K., Ohteki, T., and Takada, H. (2005) *Infect. Immun.* 73, 7967–7976
33. Viala, J., Chaput, C., Boneca, I. G., Cardona, A., Girardin, S. E., Moran, A. P., Athman, R., Memet, S., Huerre, M. R., Coyle, A. J., DiStefano, P. S., Sansonetti, P. J., Labigne, A., Bertin, J., Philpott, D. J., and Ferrero, R. L. (2004) *Nat. Immunol.* 5, 1166–1174
34. Li, J., Moran, T., Swanson, E., Julian, C., Harris, J., Bonen, D. K., Hedl, M., Nicolae, D. L., Abrahams, C., and Cho, J. H. (2004) *Hum. Mol. Genet.* 13, 1715–1725
35. van Heel, D. A., Ghosh, S., Butler, M., Hunt, K. A., Lundberg, A. M., Ahmad, T., McGovern, D. P., Onnie, C., Negoro, K., Goldthorpe, S., Foxwell, B. M., Mathew, C. G., Forbes, A., Jewell, D. P., and Playford, R. J. (2005) *Lancet* 365, 1794–1796
36. Vavricka, S. R., Musch, M. W., Chang, J. E., Nakagawa, Y., Phanvijhitsiri, K., Waypa, T. S., Merlin, D., Schneewind, O., and Chang, E. B. (2004) *Gastroenterology* 127, 1401–1409
37. Schleifer, K. H., and Kandler, O. (1972) *Bacteriol. Rev.* 36, 407–477
38. Chin, A. I., Dempsey, P. W., Bruhn, K., Miller, J. F., Xu, Y., and Cheng, G. (2002) *Nature* 416, 190–194
39. Granum, P. E., and Lund, T. (1997) *FEMS Microbiol. Lett.* 157, 223–228
40. Hosoi, T., Hirose, R., Saegusa, S., Ametani, A., Kuchi, K., and Kaminogawa, S. (2003) *Int. J. Food Microbiol.* 82, 255–264
41. Wills-Karp, M., Santeliz, J., and Karp, C. L. (2001) *Nat. Rev. Immunol.* 1, 69–75
42. Ward, P. A., and Hill, J. H. (1972) *Proc. Soc. Exp. Biol. Med.* 141, 898–900
43. Yin, E. T., Galanos, C., Kinsky, S., Bradshaw, R. A., Wessler, S., Luderitz, O., and Sarmiento, M. E. (1972) *Biochim. Biophys. Acta* 261, 284–289
44. Tanamoto, K., Zahringer, U., McKenzie, G. R., Galanos, C., Rietschel, E. T., Luderitz, O., Kusumoto, S., and Shiba, T. (1984) *Infect. Immun.* 44, 421–426
45. Proctor, R. A., and Textor, J. A. (1985) *Infect. Immun.* 49, 286–290
46. Hajjar, A. M., Ernst, R. K., Tsai, J. H., Wilson, C. B., and Miller, S. I. (2002) *Nat. Immunol.* 3, 354–359
47. Backhed, F., Normark, S., Schweda, E. K., Oscarson, S., and Richter-Dahlfors, A. (2003) *Microbes Infect.* 5, 1057–1063