Regulation of mRNA Expression in Macrophages after Yersinia enterocolitica Infection

ROLE OF DIFFERENT Yop EFFECTORS*

The Yop virulon, which comprises a complete type III secretion system and secreted proteins, allows bacteria from the genus Yersinia to resist the nonspecific immune response of the host. This virulon, which is encoded by a plasmid called pYV in Yersinia enterocolitica, enables extracellular bacteria to inject six Yop effectors (YopE, -H, -T, -O, -P, -M) into the host cell. To investigate the role of YopP, YopM, and the other pYV-encoded factors on the expression of the host cell genes, we characterized the transcriptome alterations in infected mouse macrophages using the microarray technique. PU5-1.8 macrophages were infected either with an avirulent (YopE, -H, -T, -O, -P, -M) mutant of Y. enterocolitica. Expression alterations in response to Y. enterocolitica infection were monitored for 6657 genes. Among those, 857 genes were affected, 339 of which were specifically regulated by the action of the Yop virulon. Further analysis of those 339 genes allowed identification of specific targets of YopP, YopM, or the other pYV-encoded factors. According to these results, the main action of the Yop virulon is to counteract the host cell pro-inflammatory response to the infection. YopP participate to this inhibition, whereas another pYV-encoded factor appears to also be involved in this down-regulation. Besides, YopM was found to induce the regulation of genes involved in cell cycle and cell growth, revealing for the first time an in vitro effect for YopM. In addition to YopM, other pYV factors distinct from YopP affected the expression of genes involved in cycling. In conclusion, these results provide new insight into the mechanisms of Yersinia pathogenicity by identifying the changes in host genes expression after infection and highlight the concerted actions of the different Yop effectors.

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Pathogenic bacteria have in common the capacity to overcome the defense mechanism of their animal host. Various pathogenesis mechanisms have evolved in parallel to different bacterial life styles, e.g. extracellular or intracellular. The genus Yersinia includes three species (Yersinia pestis, Yersinia pseudotuberculosis, and Yersinia enterocolitica) that are pathogenic for rodents and humans (1). Despite their different routes of infection, they are all mainly extracellular bacteria and share a common tropism for lymphoid tissues and a capacity to resist the innate immune response. This capacity is linked to the Yop virulon, a powerful weapon encoded by a virulence plasmid, called pYV in Y. enterocolitica. This plasmid encodes a complete type III secretion system and the secreted proteins (called Yop) with their chaperones (2). In addition, Y. enterocolitica possesses two adhesins, one chromosomally encoded (invasin) and one pYV-encoded (YadA), which allow a close contact with eukaryotic cells. This type III secretion system enables extracellular bacteria adhering to their host cell to insert a pore in the plasma membrane (involving LcrV, YopB, YopD) (2) and to deliver into the cell six Yop effectors (YopE, -H, -T, -O, -P, -M) (1). Although the precise role of some of the Yops is still not clear, most of them (YopE, -H, -O, -M) have been shown to be necessary for full virulence of Y. enterocolitica in the mouse infection model.

The Yop effectors can be divided into three functional groups. Four Yops (-E, -H, -T, -O) disorganize the host cytoskeleton and block bacterial phagocytosis by macrophages and polymorphonuclear leukocytes.1 YopE, YopT, and YopO (YopK in Y. pseudotuberculosis) depolymerize the actin filaments by acting on different Rho GTPases (4–6). YopH dephosphorylates and inactivates proteins associated to focal adhesion (7, 8). In addition, its role on cytoskeleton destabilization, YopH was recently shown to inhibit the phosphatidylinositol 3-kinase pathway, leading to the inhibition of T lymphocytes proliferation (10).2 YopP (YopJ in Y. pestis and Y. pseudotuberculosis) blocks the NF-κB pathway by preventing the activation of IkB kinase β. This abolishes the migration of NF-κB to the nucleus, thereby abrogating the onset of the pro-inflammatory response (11). Besides, YopP inactivates the mitogen-activated protein kinase (MAPK) pathway by inhibiting MAPK kinases (MKKs) activ-

1 Grosdent, N., Maridonneau-Parini, I., Sory, M. P., and Cornelis, G. R. (2002) Infect. Immun., in press.
2 Sauvonnet, N., Lambermont, I., van der Bruggen, I. P., and Cornelis, G. R. (2002) Mol. Microbiol., in press.
3 The abbreviations used are: MAPK, mitogen-activated protein kinase; TNF, tumor necrosis factor; SOM, self-organizing map; IL-1, interleukin 1; MIP, macrophage inflammatory protein; IL-1rn, IL-1 receptor antagonist; MCPO, monocyte chemotactic protein.
Mcrophages Transcriptome Analysis during Yersinia Infection

**EXPERIMENTAL PROCEDURES**

**Cells and Bacteria**—The bacterial strains used were the wild type *Y. enterocolitica* serotype O9 MR840 (pYV*) and its plasmid-cured derivative (called pYV) (18). The two single knockout mutants MR840 (pMKp41) (called yopP) and MR840 (pAB408) (called yopM) were described previously (19). All the strains were currently grown in brain-heart infusion at 25 °C with the appropriate antibiotics, 35 μg/mL naldixic acid, and 1 mM arsenite. The PU5–1.8 mouse macrophage-like cell line was currently cultivated in RPMI 1640 medium (Invitrogen) supplemented with 2 mM l-glutamine, 10% (v/v) fetal bovine serum (Invitrogen), 100 units/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere under 5% CO₂.

**RNA Extraction and Northern Blot Analysis**—Cytoplasmic RNAs were prepared using the Nonidet P-40 method as described (20). Briefly, cells were washed in phosphate-buffered saline (1×) and lysed in 1 mL of cold Nonidet P-40 lysis buffer supplemented with 20 mM dithiothreitol and 500 units/mL RNasin (Roche Molecular Biochemicals). Nuclei and mitochondria were removed by brief centrifugation (20 s at 10,000 × g), and proteins were digested with 200 μg/mL proteinase K (Merck) in the presence of 1% SDS and 10 mM EDTA. After phenol/chloroform extraction, RNAs were precipitated with 2.5 volumes of ethanol in the presence of 0.3 M sodium acetate. RNA quality was checked by Northern blotting and hybridization with a probe specific for β-actin mRNA.

For Northern blot analysis, RNA samples were separated by electrophoresis through formaldehyde denaturing 1.2% agarose gels and transferred onto nylon membranes (Genescreen, PerkinElmer Life Sciences) (21). RNA distribution was visualized by methylene blue staining, and Northern blots were hybridized sequentially with DNA probes synthesized by random priming using the Megaprime Kit (Stratagene) and [α-32P]dCTP. Probe templates from I.M.A.G.E. clones (I.M.A.G.E. clones 1139544, 5325073, 5738898, and 4486098) were generated by insert digestion and purification after verification of the clone identity by sequencing. Hybridizations were performed overnight at 42 °C followed by washes as described (21), and the last wash was performed at 65 °C for 30 min in 0.1× SSC (1× SSC = 0.15 M NaCl and 0.015 M sodium citrate), 1% SDS. Signals were detected and quantified by phosphorimaging (Molecular Dynamics, Sunnyvale, CA).

**cRNA Synthesis, Affymetrix GeneChip Probe Array Hybridization, and Data Analysis**—For cRNA synthesis, 30 μg of cytoplasmic RNAs from either non-infected or infected PU5–1.8 were used as templates for double-stranded cDNA synthesis using the SuperScript Choice System ( Invitrogen) and a T-dT24 primer according to Affymetrix instructions. After purification, double-stranded cDNAs were used as template for in vitro Tₐ transcription using the Bioarray high yield transcription labeling kit (Enzo). cRNA synthesis yields, as monitored by spectrophotometry, were highly similar in the different samples (typically 8–10 μg of cRNA/mL used, corresponding to roughly a 500-fold amplification), 20 μg of each cRNA population were fragmented. First, 5 μg were used as target for the Te3 test chip to check the quality of the target. Subsequently, 15 μg were used for hybridization of the murine genome GeneChip probe arrays U74Av2 (both arrays from Affymetrix). Hybridization, washes, antibody amplification, and staining were performed using the Affymetrix fluidics station and scanner following the manufacturer’s instructions. Analysis of the raw data was performed using Affymetrix Suite software. Further analysis were performed using the Excel and GeneCluster (available at www.genome.wi.mit.edu/MPR/software.html) software.

**RESULTS AND DISCUSSION**

**Preliminary Considerations**—To characterize the specific effects of the *Y. enterocolitica* Yop virulon on the host cell transcriptome, we analyzed gene expression of infected PU5–1.8 murine macrophages. A preliminary kinetic analysis showed that a 2.5-h infection with wild type (pYV*) *Y. enterocolitica* was sufficient to elicit a robust effect on the inflammatory response, as monitored by tumor necrosis factor α (TNF-α) mRNA level and induced less than 10% of apoptosis, as monitored by propidium iodide incorporation (data not shown). Because one early effect of apoptosis induction is the degradation of cellular mRNAs, this point was critical to ensure extraction of high quality, undegraded cytoplasmic RNAs.

We analyzed mRNA expression from macrophages infected for 2.5 h with pYV*, pYV**, yopP*, and yopM* bacteria, and non-infected PU5–1.8 macrophages. After infection, cytoplasmic RNAs were extracted to monitor the variations of mature mRNAs by the Affymetrix GeneChip system. We used the oligonucleotide probe array MG-U74Av2, which allows measurement of the expression levels of about 6000 mRNAs and 6000 Expressed Sequence Tags (EST; for simplification, we use the word “gene” indifferently for known genes or EST). In each experiment, the number of mRNA species detected (called “present” by the Affymetrix Suite analysis software) was about 40–46%, and the analysis was carried out on 6657 genes. In every experiment, the mean signal intensities were arbitrarily adjusted to 150. The dynamic range of the data as well as the signal distributions were very similar, encompassing more than 4 orders of magnitude (Fig. 1), hence validating the reliability of the microarray experiments. According to those distributions, the background was empirically set to 20 so that the genes with data values below this threshold were considered to have undetectable mRNA expression levels. Furthermore, Affymetrix array values for most of the so-called “housekeeping genes” such as β-actin and elongation factor 1α (*EF1α*) were found to be similar in the five conditions, coherent with the results obtained by Northern blot hybridizations (Fig. 2). Those Northern blots were performed with RNA from three different infection experiments to check the reproducibility of the results.

**Genes Regulated by PU5–1.8 Macrophages in Response to the Bacterial Infection, Independently of the Yop Virulon**—First we compared the microarray hybridization data from the non-

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4 J. del Prete, M. S. Robles, A. Guio, C. Martinez-A, M. Izquierdo, and J. A. Garcia-Sanz, submitted for publication.
infected PU5–1.8 cells with those from cells infected with pYV− or pYV+ bacteria. Genes were considered as not regulated and, hence, were discarded from the analysis if they did not show any significant variation of their expression level in the cells infected by the pYV− or the pYV+ bacteria, as compared with the non-infected cells (i.e., ratio values <2 or difference between the values <30). This yielded a data set of 857 genes regulated upon Y. enterocolitica infection, representing nearly 13% of the 6,657 genes analyzed. To cluster genes according to the variation pattern of their mRNA level, self-organizing maps (SOMs) analyses were performed using the GeneCluster software (23), resulting in the identification of eight different groups of genes (Fig. 3 and www.biozentrum.unibas.ch/cornelis/manuscript/Folder_1.html).

The first two clusters, called A and B, gathered 518 genes that were similarly expressed in cells infected with pYV− and pYV+ bacteria but differently in the non-infected cells (Fig. 3). Thus, these variations represented the PU5–1.8 macrophage response to the bacterial infection, which was not modulated by the Yop virulon. Supporting this interpretation, we found numerous genes encoding cytokines and other pro-inflammatory proteins in the cluster A, which gathers genes up-regulated in pYV− and pYV+-infected cells (see Folder 1). These included the small inducible cytokines A3 (SCYA3; J0BB91), A5 (SCYA5; AF0659B7), and A6 (SCYA6; M5800B), interleukin 1 (IL-1) α and β (M1B639 and M15151), and the macrophage inflammatory proteins 1b and 2 (MIP-1b, X62502; MIP-2, X53798), most of which are up-regulated after infections with bacteria as diverse as Escherichia coli, Listeria monocytogenes, Bordetella pertussis, or Salmonella (17, 24–26). As an example, Northern blot analysis of IL-1β mRNA expression was in complete agreement with the microarray data (Fig. 2). Besides, cluster A enclosed less predictable factors also involved in immune response, such as the complement component C3 (K02782), which plays a central role in phagocytosis (27), and Fas receptor (M836B9), which is up-regulated in macrophages after activation, as a mechanism of negative feed-back (28). In cluster B, corresponding to the cluster A-inverted pattern, we found numerous genes encoding intracellular signaling proteins among which the diacylglycerol kinase α (GenBank™ AF085219). Diacylglycerol kinase α is involved in phosphatidylinositol triphosphates synthesis and, although it has long been considered as a housekeeping gene, recent analysis in mice reveals that diacylglycerol kinase α mRNA is down-regulated after T lymphocyte activation.5

5 M. A. Sanjuán, B. Pradet-Balade, D. R. Jones, C. Martínez-A, J. A. García-Sanz, and I. Mérida, submitted for publication.
A previous study of the neutrophil transcriptome during Y. pestis infection showed that some cytokines (namely, SCYA3, MIP-2, IL-1α and -β) are up-regulated in the pCD1 (pYV, analogous)-infected neutrophils but not in the pCD1+ (pYV+ analogous) ones (17). This result contrasts with our finding that those genes were up-regulated in both Y. enterocolitica pYV− and pYV+-infected macrophages (cluster A, Fig. 3). This discrepancy can be explained by differences between the two infected cell types (human neutrophils versus mouse macrophage cell line). Indeed, the same authors show transcriptome variations between monocytes and neutrophils infected by E. coli (17). This divergence could also result from the fact that two different Yersinia species were used (Y. pestis versus Y. enterocolitica). In fact, at least for IL-1α, it was recently reported that its gene was up-regulated in macrophages infected by wild type Y. enterocolitica (30). Finally, because 60% of the identified regulated genes belong to clusters A and B, the majority of the alterations in gene expression that take place in Y. enterocolitica-infected cells represents the macrophage response to the bacterial infection independently of the Yop virulon (Fig. 3).

Identification of Genes Whose Expression Is Modified by the Yop Virulon—The six other clusters (C–H) regrouped 339 genes whose mRNA levels differed in cells infected by the pYV+ from that infected by the pYV− bacteria (Fig. 3 and www.biozentrum.unibas.ch/cornelis/manuscript/Folder_2.html). Those genes were, thus, specifically regulated by factors encoded by the Y. enterocolitica pYV plasmid. Strikingly, the majority of these genes were found in clusters C and F (198 on 339, i.e., 58% of the pYV-regulated genes). These two clusters gathered genes up (F)- or down (C)-regulated after pYV+ infection but on which the pYV-encoded factors induced a deregulation so that the mRNA expression level in pYV+-infected cells was similar to that in the non-infected cells (Fig. 3). This most likely represents a specific mechanism by which Y. enterocolitica counteracts the macrophage activation, by shutting down the host cell response. Because most of the genes identified as specifically regulated by the Yop virulon belong to this category, one of the most important functions accomplished by the pYV-encoded factors seems to be to counteract the host cell response to the infection. This is in agreement with previous observations made on neutrophils infected by Y. pestis, where more genes showed altered expression in pYV+-infected cells than in pYV−-infected ones (17).

To gain further insight into the mechanism by which the Yop virulon regulates the host cell transcriptome, PU5–1.8 cells were infected with the Y. enterocolitica yopP− and yopM− strains in which the genes encoding YopP or YopM have been knocked out. mRNA expression of the 339 genes previously identified as specifically regulated by pYV-encoded factors (clusters C–H) were analyzed in non-infected cells and cells infected with the four Y. enterocolitica strains. Again, SOMs were generated to cluster genes following their pattern of mRNA expression. This analysis yielded as many as 24 different clusters, some of which could not be interpreted by the sole effect of YopP or YopM or of the other pYV-encoded factors. For this reason, we decided to concentrate on the patterns that undoubtedly displayed the effect of either (i) YopP, (ii) YopM, or (iii) the other factors encoded by the pYV plasmid. This global analysis, taking simultaneously into account the five different conditions, is more stringent than the comparison of expression profiles two by two (i.e. pYV− versus yopP−) and, thus, should yield more reliable results concerning the target genes controlled by the different Y. enterocolitica effectors.

Specific Changes of Host Gene Expression Dependent on YopP—Genes were considered as specifically regulated by YopP action if they were expressed at similar levels in pYV+ and yopM−infected cells on one side and in pYV− and yopP− infected cells on the other side. Four clusters (I, J, K, L), which represent 59 genes, fulfill this criterion (Fig. 4, Table I, www.biozentrum.unibas.ch/cornelis/manuscript/Folder_2.html). Fig. 2 shows the Northern hybridization of two YopP targets, TNF-α and TNF receptor 2, which expression correlates with the profiles determined by Affymetrix chip hybridization.

For most of the genes identified as YopP targets (clusters I + K = 37 genes), the effect of YopP consisted in restoring the mRNA expression to the level observed in non-infected cells. The majority of those genes are involved in the immune response, consistent with the known capacity of YopP to inhibit the inflammatory response (31). This is particularly the case for cluster I, which represents all the genes that were up-regulated in pYV− and yopP−-infected cells as compared with the non-infected cells and the pYV+ and yopM−-infected cells (Fig. 4). These genes code for cytokines (TNF-α), receptors (TNF receptor 2), signaling molecules (Traf1), apoptotic modulators (A20), or transcription factors modulators (junB, IκBα), many of which are involved in the TNF-α-signaling pathway (Table I) (32). Strikingly, nearly all the genes from this cluster were shown to be inducible by NF-κB (33) (Table I). Their down-regulation may thus result from the inhibition exerted by YopP on IκB kinase β, which leads to the inhibition of NF-κB activation (12). Thus, these results, coherent with the known action of YopP, further support the reliability of our microarray data and demonstrate for the first time that mRNA expression of those genes is actually down-regulated by the action of YopP in Y. enterocolitica-infected macrophages. Conversely, it should be noted that not every potential NF-κB target induced by pYV− infection appeared to be down-regulated by YopP (example, IL-1β, MIP-2). This may be explained either by a rather high stability of their mRNAs, whose level decreased later than that of TNF-α mRNA for instance or by the induction of those mRNAs by transcription factors other than NF-κB. This difference between YopP actions on the distinct NF-κB targets illus-

![Image](68x526 to 278x729)

**Fig. 3.** Expression patterns of PU5–1.8 macrophage genes regulated after Y. enterocolitica infection. 887 genes whose expression was at least 2-fold different between non-infected cells (N) and cells infected by Y. enterocolitica pYV− (-) or pYV+ (+) were clustered by SOM analysis according to their expression profile into 8 groups (A–H).

The number of genes related to each cluster is indicated above. The relative contribution of the different clusters A + B, C + H, E + F, and D + G to the total regulations is represented schematically by a pie diagram.
tractates the complexity of the cellular regulations, and the need for such a wide analysis to unravel the targets of a Yop effector.

Another interesting target from cluster I is eIF2B, a key regulator of protein translation (34), hence suggesting that YopP might inhibit protein synthesis. Besides, eIF2B activity was shown to be induced by the MAPK pathway (35), a cascade that is itself inactivated by YopP (12). As a result, YopP might decrease eIF2B protein and activity through acting at both the transcriptional and the post-translational levels. Because the inhibition of eIF2B activity was recently shown to induce apoptosis (36), those effects might be connected with the pro-apoptotic function of YopP (19).

Cluster K, corresponding to cluster I inverted pattern, contained genes that were up-regulated specifically by the action of YopP (Fig. 4). Again, this cluster was rather homogenous as it gathered mainly genes encoding cell surface proteins (Table I).

In contrast with the previous clusters, clusters J and L gathered genes for which YopP induced a marked difference in their expression level as compared with those observed in non-infected cells. Thus, the action of YopP is not only to restore the expression level to that in the non-infected cells. For example, genes from cluster L were up-regulated in yopP + infected macrophages on one side and in pYV - and yopP -infected cells on the other side (Fig. 4). For half of them (clusters N + O = 13 genes), YopM induced a change in their expression level as compared with those observed in the non-infected cells (Fig. 4).

Most of the genes regulated by YopM action seemed to be specifically regulated by YopM action if they shared similar mRNA expression levels in pYV - and yopM -infected macrophages on one side and in pYV + and yopP + infected cells on the other side (Fig. 4, Table II, www.biozentrum.unibas.ch/corne-lis/manuscript, Folder 2). This led to the identification of 25 genes, organized into three clusters (M, N, and O, Fig. 4). For half of them (clusters N + O = 13 genes), YopM induced a change in their expression level as compared with those observed in the non-infected cells (Fig. 4).

Most of the genes regulated by YopP action seemed to be related to the cell cycle and cell growth (Table II). These include genes encoding proteins involved in DNA replication and repair such as the DNA mismatch repair protein (MLH1, cluster M) and the checkpoint kinase 2 (CHK2, cluster N), both of which belong to the genome surveillance network. A defect of these genes can lead to aberrant cell growth (41, 42). Caf-1 (cluster O), which also participates to the general DNA surveillance, is involved in chromatin assembly and DNA replication (43). These three genes, involved in DNA maintenance, were all down-regulated by YopM action (Table II). Furthermore, the transcription factor B-myb (cluster M) was also down-regulated MAPK. Genes from cluster J were down-regulated in a YopP-dependent way. These include PCMT1 and PNAD, both implicated in protein repair and degradation (39), together with procollagen type IV and junction plakoglobin, involved in cell-extracellular matrix and cell-cell cohesion (40) (Fig. 4). Hence, this cluster shows again some homogeneity with regard to the gene functions and describes new targets of YopP. In conclusion, our analysis points out the importance of YopP in the inhibition of the host cell response and allows direct identification of new targets of this virulence factor.

Specific Changes of Host Gene Expression Dependent on YopP—As described for YopP targets, genes were considered as specifically regulated by YopM action if they shared similar mRNA expression levels in YopM - and yopM -infected macrophages on one side and in YopM + and yopP +infected cells on the other side (Fig. 4). Most of these genes encode cell surface proteins involved in the inhibition of the MAPK pathway (Fig. 4). Among those genes are MAPK phosphatase (MKP-1) and G protein signaling regulator (RGS2) (Table I). Both proteins are stress-induced, and both have been implicated in the inhibition of the MAPK pathway (37, 38). It has been reported that YopP inhibits MAPK by directly inactivating their upstream kinases (12). Our results suggest the existence of additional, yet undescribed mechanisms by which YopP could inhibit the MAPK signaling pathway; that is, by inducing the overexpression of mRNAs encoding proteins able to inhibit MAPK.

Fig. 4. Expression patterns of PU5-1.8 macrophage genes regulated by the action of YopP, YopM, or pYV-encoded factors distinct from YopP and YopM. The expression profiles of 339 pYV-regulated genes were analyzed in PU5-1.8 either non-infected (N) or infected by Y. enterocolitica pYV (-), YopP + (P+), and yopM + (M+). SOM analysis allowed identification of genes specifically regulated by the action of (i) YopP, gathered into four clusters (I-L), (ii) YopM, gathered into three clusters (O–M), and (iii) pYV-encoded factors distinct from YopP or YopM, gathered into five clusters (P–T).
by the action of YopM. B-myb is a cell cycle regulator that stimulates hematopoietic cell proliferation and whose expression is itself regulated through the cell cycle, being repressed during G0/early G1 phases, induced in late G1, and maximal in S phase (44). In addition to those cell cycle modulators, YopM affects factors involved in cell growth.

In conclusion, YopM targets appear to have cellular functions very distinct from those of YopP targets or from those of

In addition, YopM induced the down-regulation of the expression of at least two signaling proteins involved in cell growth. Trio (cluster M) regulates cytoskeleton reorganization by activating Rho/Rac GTPases and is necessary for cell migration and growth (49). P52rIp (cluster M), although its function is not yet well defined, has been implicated in the indirect activation of protein kinase R, an inhibitor of stressed cell growth (49). This shows that, in addition to cell cycle regulators, YopM affects factors involved in cell growth.

In addition, calystemin 1 (cluster O) and Mac-1s (cluster O) were both up-regulated by YopM action. These two factors are involved in cell adhesion. In particular, Mac-1s is the C3 receptor involved in bacterial phagocytosis, thereby playing a crucial role in innate immunity (50). Although the biological meaning of these up-regulations remains obscure, they are obviously related neither to cell cycle nor to cell growth, and they represent another class of YopM target genes.

In conclusion, YopM targets appear to have cellular functions very distinct from those of YopP targets or from those of

by the action of YopM. B-myb is a cell cycle regulator that stimulates hematopoietic cell proliferation and whose expression is itself regulated through the cell cycle, being repressed during G0/early G1 phases, induced in late G1, and maximal in S phase (44). In addition to those cell cycle modulators, YopM targets included genes encoding proteins involved in intracellular trafficking, which could also play a role during the cell cycle. PLD3 (cluster M) is implicated in plasma membrane traffic (45), whereas STB2 (cluster N) has been involved in the traffic from the Golgi apparatus to the plasma membrane (46). In contrast, P3D3 and STB2, which were both down-regulated, dynein mRNA expression (cluster O) was up-regulated by YopM action. Dynein is a microtubule motor that plays a central role in vesicular transport and in chromosome movement. Dynein has recently been shown to be up-regulated during cellular senescence, suggesting a role for dynein as a tumor suppressor (47). Altogether these data suggest that YopM affects several cellular functions that may ultimately perturb cell division.

In conclusion, YopM targets appear to have cellular functions very distinct from those of YopP targets or from those of

| Accession No. | Description | Cluster |
|---------------|-------------|---------|
| D84196        | Tumor necrosis factor α (TNF-α) | I       |
| AJ01967       | Growth/differentiation factor 15 (GDF-15, SBP) | I       |
| X87128        | p75 TNF receptor (TNFR2) | I       |
| M89641        | Interferon α/β receptor (IFN α/βR) | I       |
| U59430        | Neuropeptide_Y receptor Y6 (NPY6R) | K       |
| Cell surface protein | | |
| AF030454      | Epithelial V-like antigen (EVA) | K       |
| X00652        | Ig-kappa light chain V-J-κ | K       |
| AI843313      | Glypican-3 precursor (GPC3) | K       |
| AF016308      | Histocompatibility 2, M region locus 9 (H2-M9) | K       |
| Signaling | | |
| L07924        | Guanine nucleotide dissociation stimulator for Ras-related GTPase (Rgs) | I       |
| L35302        | TNF receptor-associated factor1 (TraF1) | I       |
| U88328        | suppressor of cytokine signaling-3 (SOCS-3) | I       |
| AF013490      | Protein-tyrosine phosphatase (MEG2) | J       |
| AV226788      | Dual specificity tyrosine phosphatase 8 (DUS8) | K       |
| X61940        | Growth factor-inducible immediate early (MKP-1) | L       |
| U67187        | G protein signaling regulator (RGS2) | L       |
| Transcription | | |
| U20735        | Transcription factor junB | I       |
| AF017021      | Basic leucine zipper transcription factor, ATF-like (B-ATF) | I       |
| U37524        | I kappa B alpha (1κBα) (κBα) | I       |
| X76858        | E4F transcription factor 1 (E4F1) | K       |
| U60453        | Enhancer of zeste homolog 1 (Ezh1) | L       |
| Growth arrest and apoptosis | | |
| U19463        | Tumor necrosis factor-induced protein 5 (A20, TN Flp3) | I       |
| L24118        | Tumor necrosis factor-induced protein 2 (TNFflp2) | I       |
| U60697        | Growth arrest and DNA damage-induced 45 (GADD45) | I       |
| X51829        | Myeloid differentiation primary response (Myd116) | I       |
| Protein synthesis/degredation | | |
| U79962        | TAR RNA-binding protein 2 (TARBP2) | I       |
| M98036        | Guanine nucleotide exchange factor delta (eIF2B) | I       |
| M60292        | Protein-1-isosapartate (n-isapartate) O-methyltran ferase1 (PCMT1) | J       |
| U57692        | N-terminal Asn amidohydrolase (PNAD) | J       |
| Intracellular | | |
| AI952541      | Tripartite motif protein 13 (Trim13) | I       |
| AF033473      | kinesin heavy chain (KIF5B) | K       |
| Metabolism | | |
| AV174870      | Aspartyl aminopeptidase (Dnpep) | I       |
| X61147        | Aconitase 1(tra1, Aco-1) | K       |
| AI843947      | ATP synthase mitochondrial (ATP5J2) | K       |
| Y15733        | 17-β-hydroxysteroid dehydrogenase type 7 (HSD17b7) | L       |
| M37831        | Cytochrome c oxidase, subunit IV (COXIV) | L       |
| ECM/cytoskeleton | | |
| AF041448      | Advillin (DOC6) | I       |
| M15832        | Procollagen, type IV (Col4a-1) | J       |
| M90365        | Junction plakoglobin (Pcj) | J       |
the other pYV-encoded factor targets (see below), as none of those genes is involved in the pro-inflammatory response. Although YopM clearly contributes to virulence (15), its role from in vitro experiments remained elusive. This represents the first approach in which target genes responsive to YopM have been identified. The mechanism by which YopM affects these target genes remains to be unraveled. Because YopM is found in the cell nucleus (16), it could interact directly with proteins involved in mRNA metabolism (i.e. transcription, maturation, export, etc.) to regulate mRNA expression.

Specific Changes of Host Gene Expression Dependent on pYV-encoded Factors Distinct from YopP or YopM—A total of 107 genes (representing 32% of the pYV-regulated genes) had similar expression levels in pYV−, yopP−, and yopP−infected cells but different expression levels in the pYV−infected cells. Thus, these genes appear to be regulated by pYV-encoded factors other than YopP or YopM. By SOM analysis, these genes were grouped according to their expression patterns into five different clusters (P, Q, R, S, T) (Fig. 4, Table III, www.biozentrum.unibas.ch/cornelis/manuscript, Folder 2).

Cluster R regrouped genes that were only up-regulated in the pYV−infected macrophages, so that some pYV-encoded virulence factors restored their expression to the level observed in non-infected cells. This cluster groups a set of genes involved in the inflammatory responses. These include cytokines (granulocyte colony-stimulating factor, MCP-1, MCP-3) and their modulators (IL-1RN) as well as other proteins generally induced after bacterial infection, such as RGS16 and urokinase-type plasminogen activator (51, 52) (Fig. 4, Table III). The expression of these genes was down-regulated by the action of pYV-encoded proteins other than YopP. Indeed, Northern blot hybridizations confirmed that mRNA expression of MCP-1 and IL-1RN, taken as two examples, were actually down-regulated in the pYV−, yopP−, and the yopP−infected cells, as compared with the pYV−infected ones (Fig. 2). This finding reveals that, in addition to YopP, at least another virulence factor contributes to the inhibition of the pro-inflammatory response. LcrV is a pYV-encoded factor with multiple functions. It may represent one of the candidates responsible for the control of pro-inflammatory response genes, since long term preincubation of macrophages with purified LcrV has been shown to induce IL-10 secretion, which in turn prevents cytokine release in activated macrophages (53). However, the fact that IL-10 gene was not expressed under any of the 5 conditions analyzed in this study raises doubts about the possible control of the genes from cluster R by LcrV. Another candidate is YopH, which has been recently demonstrated to suppress the activation of the phosphatidylinositol 3-kinase induced upon Y. enterocolitica infection. Phosphatidylinositol 3-kinase is known to induce different pathways that can lead to the up-regulation of inflammatory genes such as MCP-1 and urokinase-type plasminogen activator (54–56) so that its inhibition by YopH could then down-regulate the inflammatory genes from the cluster R. These results reveal that Y. enterocolitica overcomes the pro-inflammatory response through different mechanisms exerted by distinct pYV-encoded factors.

The cluster Q regrouped genes that were specifically up-regulated by a pYV-encoded factor other than YopP and YopM. It appeared to be very homogeneous with regard to the function of the genes, in majority transcription factors (Table III). Among these, five genes belong to the family of the Kruppel-like transcription factors (Klf9, Klf4, Klf2, Zpf94, and Zpf36), suggesting that a Y. enterocolitica pYV-encoded factor affects a common regulator of this family. These Kruppel-like factors are involved in differentiation, development, and cell growth arrest (especially Klf4 and Klf2), and they can be induced by cytokines or cell injury (57). Up-regulation of these transcription factors in pYV−infected cells may, thus, play a role in Y. enterocolitica pathogenesis. Furthermore, cluster Q enclosed other transcription factors that can be involved in cell cycle arrest such as c-Jun and Dmp1 (22, 29). Hence, in addition to YopM, other pYV-encoded factors seem to affect the expression of host cell growth regulators. This illustrates that Y. enterocolitica virulence factors act in a concerted way and may explain why no clear effect of YopM has been identified so far.

Clusters S and T, which both contained genes down-regulated by the pYV-encoded factors, seem to affect the expression of host cell growth regulators. This illustrates that Y. enterocolitica virulence factors act in a concerted way and may explain why no clear effect of YopM has been identified so far.

### Table II

| Accession No. | Description | Cluster |
|---------------|-------------|---------|
| AA920621      | DNA mismatch repair (Mih1) | M       |
| AF008905      | Checkpoint kinase 2 (Chk2, chek2, rad 53) | N       |
| AJ152771      | Chromatin assembly factor-1 p150 (Caf-1) | N       |
| AV214934      | Myb-related protein B (B-myb) | M       |
| AF026124      | Phospholipase D3 (PLD3) | M       |
| AF063299      | Dynamin (DH IC-1, DNIC11, DNIC11) | O       |
| AV365041      | Syntaxin-binding protein2 (STB2) | N       |
| AB030621      | Choline/ethanolamine kinase α (CK/EK) | M       |
| AI859702      | Copper chaperone for superoxide dismutase Ccsd | O       |
| AI852548      | Peroxisomal membrane protein (PFMP34) | O       |
| AF093870      | T cell receptor β V-D-J region (TCR) | M       |
| AI847699      | Calcyntenin 1 (Cstn1) | O       |
| X07640        | Cell surface glycoprotein (Mc-1, CD11b, CR3) | O       |
| AA879937      | Repressor of inhibitor of protein kinase R (P52rPK) | M       |
| AA110657      |Triple functional domain protein (Trio) | M       |
| AF068199      | n-dopachrome tautomerase | M       |
| AI854725      | Placental 6 protein (PL6) | M       |
| M37759        | Serine 1 ultra high sulfur protein | O       |
lated by a pYV-encoded factor, enclosed several genes encoding proteins involved in signaling pathways such as the MAPK (Pip92, A-raf), the Ras pathway (rap2B), or even the phosphatidylinositol 3-kinase cascade (p85 subunit) (Fig. 4, Table III). It was recently reported that the phosphatidylinositol 3-kinase could regulate the mRNA expression of its own p85 subunit (9) so that the alteration of p85 mRNA expression could result from YopH action on the phosphatidylinositol 3-kinase. In addition, YopE, -H, -T, and -O affect Rho GTPase and focal adhesion proteins at the post-translational level. Because cross-talks between Ras, Rho, and MAPK signaling pathways have been described (3), YopE, -H, -T, -O might also be involved in the mRNA regulation of some of those cascades players.

The analysis of the host cell transcriptome reveals that the

| Accession No. | Description | Cluster |
|---------------|-------------|---------|
| L22938        | Interleukin 1 receptor antagonist (IL-1rn) | R       |
| M13928        | Colony-stimulating factor, granulocyte (G-CSF) | R       |
| M19681        | Small inducible cytokine A2 (SCYA2, MCP-1) | R       |
| X70658        | Small inducible cytokine A7 (SCYA7, MCP-3) | R       |
| M14872        | Gonadotropin-releasing hormone (GnRH-GAP) | P       |
| M95200        | Vascular endothelial growth factor (VEGF) | P       |
| U77630        | Adrenomedullin (ADML) | P       |
| AW049031      | Immediate early response, erythropoietin 1 (FM2) | Q       |
| AB007696      | Prostaglandin E receptor (EP2) | R       |
| AB030894      | Olfactory Receptor 83 (MOR83) | Q       |
| AI840577      | Histocompatibility 2, class II antigen E (H-2Eb1) | T       |
| AF041054      | E1B 19K/Bcl-2-binding protein homologue (Nip3) | P       |
| M13071        | Raf-related oncogene (A-raf) | S       |
| M59821        | Factor-inducible protein (pigp92) | S       |
| U94829        | Regulator of G protein signaling (Rgs16) | R       |
| AI852011      | Phosphatidylinositol 4-kinase (PI4K) | R       |
| U09963        | 129 tyrosine-threonine dual specificity phosphatase (PAC-1) | R       |
| X99693        | RhoB | Q       |
| D28577        | Protein Kinase C (PKC) | Q       |
| D31842        | Tyrosine phosphatase (PTP36) | Q       |
| Y13569        | Phosphatidylinositol 3-kinase p85 (PI3K p85) | P       |
| AI837726      | RAS oncogene family member (Rap2B) | T       |
| L14543        | SH3-binding protein (SH3 BP2) | T       |
| M31885        | Inhibitor of DNA binding 1 (d1) | S       |
| AI247277      | Nef-associated factor (ABIN) | R       |
| AI848050      | Kruppel-like factor 9 (KLF9, BETB-1) | Q       |
| U20344        | Kruppel-like factor (KLF4, GKLF) | Q       |
| U20966        | Kruppel-like factor (KLF2, LKLF) | Q       |
| U97966        | Zinc finger protein (Zfp94) | Q       |
| X12761        | c-Jun | Q       |
| X14678        | Zinc finger protein 36 (Zfp36) | Q       |
| U70017        | Cyclin D-interacting myb-like protein (Dmp1) | Q       |
| U38148        | NFIL34EBP4 transcription factor | Q       |
| AW049299      | General control of amino acid synthesis-like 2 (PCAF-B/GCN5) | T       |
| U63387        | Chromobox homologue 4 (MPc2) | T       |
| M22115        | Homebox A1 (Hox-1.6) | R       |
| Y10339        | Dihydropyrimidinase-like 2 (Ulip2) | P       |
| X25774        | Maloney leukemia virus (Mov-10) | S       |
| U27457        | Origin recognition complex protein 2 homologue (ORCZL) | R       |
| U95826        | Cyclin G2 (CCNG2) | Q       |
| D29678        | Cyclin dependent kinase-5 (Cdk5) | T       |
| D29797        | Syntaxin 3 (Stx3) | R       |
| AI854462      | Ras-related protein (rab 20) | R       |
| AW122706      | Solute carrier family 8 member 7 (LAT2) | R       |
| M26887        | Amino acid transporter cationic 1 (Atrc1) | Q       |
| AI853172      | Udpglucose ceramide glucosyltransferaselike (Ep s21) | R       |
| L34570        | Arachidonate 15-lipoxygenase (12-LO) | R       |
| AF043514      | Phosphomannomutase (Pmm2) | S       |
| AI853083      | Pseudouridine synthase 1 (MPSU1) | R       |
| M17922        | Urokinase-type plasminogen activator protein (uPA) | R       |
| AF049124      | Neuronal pentraxin 2 (Nptx2) | Q       |
| V00830        | Keratin complex 1 acidic gene 10 (K10) | T       |
pYV-encoded factors other than YopP and YopM are responsible for the down-regulation of some pro-inflammatory and cell growth regulator genes. Furthermore, the induction of mRNA expression for different members of a transcription factor family suggests that this effect plays an important role in *Y. enterocolitica* pathogenesis.

**Conclusion**—The identification of the genes affected by the different *Y. enterocolitica* virulence factors by mRNA profiling allowed new insights into the mechanism of action of this bacterial pathogen. In this analysis, target genes were grouped into clusters by SOM analysis according to their expression patterns. Many of those clusters showed homogeneity with regard to the genes they contained. This observation further corroborates the relevance of our analysis and allows a functional interpretation of the data.

The results described herein demonstrate that an important role of the pYV-encoded factors on the host cell transcriptome is to counteract the regulations exerted in response to the infection. As expected, this effect is due in part to YopP, very likely through the inhibition of the NF-κB cascade. However, our results indicate that at least another pYV-encoded factor participates to the down-regulation of several genes involved in the onset of inflammation. Besides, our results reveal that YopM also modulates macrophage gene expression. Unlike the other pYV-encoded factors, YopM does not seem to affect the inflammatory response; rather, it alters the mRNA expression of genes involved in the control of the cell cycle and growth regulators. So far, this is the first in vitro effect described for YopM on the biology of the infected cell. In addition to YopM, other pYV-encoded factors (distinct from YopP) may also disturb the mRNA expression of growth regulators.

Finally, this analysis points out the cooperative effects between the various factors encoded by the Yop virulon. Concerted actions have been previously demonstrated between YopE, -H, -T, and -O to avoid bacterial phagocytosis by macrophages.¹ Our analysis reveals that, in addition, a cooperative scheme has evolved to shut down the host inflammatory response involving not only YopP but also another pYV-encoded factor. Besides, different Yops seem to be involved in the inhibition of host cell growth, YopM and other pYV-encoded factors by their effect on mRNA expression level of growth and cycle regulators. As a consequence, this analysis enabled the identification of genes regulated by the Yop virulon and the high complexity of *Y. enterocolitica* pathogenesis.

This analysis constitutes the first case where the use of different mutated *Y. enterocolitica* strains together with SOM analysis enabled the identification of genes regulated by the action of distinct virulence factors. Those genes may constitute potential therapeutic targets. This analysis provides new clues on the role of the Yop virulon, which will help to characterize the mechanisms employed by each Yop in this process.

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