Clostridia are anaerobic bacteria, although many species can tolerate oxygen to various extents. They are able to form endospores and are not capable of dissipatory sulfate reduction. Most of them show a positive Gram reaction. These criteria have been used in the past for classification. However, phylogenetic analyses based on 16S rRNA sequences led to reattribution of many former clostridia to numerous other and also novel genera, such as Blautia, Butyribrio, Caloramator, Cellulosilyticum, Dendrosporobacter, Eubacterium, Filifactor, Flavonifractor, Moorella, Oxalophagus, Oxobacter, Paenibacillus, Thermoanaerobacter, Thermoanaerobacterium, Sedimentibacter, Sporohaemobacter, Syntrophomonas, Synthrophospora, and Tissierella (J. P. Euzéby, List of prokaryotic names with standing in nomenclature – genus Clostridium, http://www.bacterio.cict.fr/c/clostridium.html). For the genus Clostridium, approximately 180 species have been validly described, rendering it one of the largest bacterial genera. Only a few of these species are pathogenic, however, involving microbes producing very dangerous toxins. On the other hand, a large number of species are used in biotechnological applications (enzyme, bulk chemicals, and biofuels production) and tested for use in cancer therapy. This is due to the enormous metabolic diversity within the clostridia, rendering them the avant-garde of biotechnologically exploited microorganisms. During past years, techniques have been developed that allowed establishment of genetic systems for many clostridia. Thus, the tools are at hand for further elucidation and
exploitation. Due to the limited space of this article, many aspects cannot be presented in detail. Thus, the interested reader is referred to recent references for additional information (1–6).

**PHYSIOLOGY OF CLOSTRIDIUM**

Clostridia are often differentiated by performing a saccharolytic or a proteolytic metabolism. However, such traits can also be present in a single organism. For example, *Clostridium tetani*, originally considered to be a paradigm of a proteolytic microbe, was reported to ferment glucose (7). Genome sequencing confirmed that this organism contains all genes required for glycolysis. Only polysaccharides cannot be degraded (8). In clostridia, there are a number of different pathways for substrate utilization, yielding one or several dominant fermentation products (Table 1).

**Homoacetate Fermentation**

This metabolic pathway has been mainly elucidated with the former *Clostridium thermoacetaticum*, now reclassified as *Moorella thermoacetica*. However, there are true clostridia that use this type of fermentation. It functions during the degradation of sugars as well as under autotrophic conditions. The sequence of enzymatic reactions leading to acetate formation from CO$_2$ plus H$_2$ or from CO is called the Wood-Ljungdahl pathway and the organisms using it are called acetogens, respectively. Clostridia, able to grow heterotrophically on sugars and autotrophically on CO$_2$/H$_2$ gas mixtures or CO, are, e.g., *C. aceticum* and *C. ljungdahlii*. *C. aceticum* was originally isolated in 1936 (9) and thought to be lost during World War II. It was rediscovered in 1981 as an old spore preparation in the laboratory of Horace A. Barker at the University of California in Berkeley (10). As *M. thermoacetica*, *C. aceticum* contains cytochromes (11). It is thus highly likely that energy conservation is achieved by generating a proton gradient over the membrane and using it for ATP synthesis via an F$_1$F$_0$-type ATPase. A model, showing a possible electron transfer chain with involvement of cytochrome, menaquinone, flavoproteins, flavodoxin, ferredoxin, and rubredoxin has been proposed by Das and Ljungdahl (12) for *M. thermoacetica* and might reflect as well the metabolism of *C. aceticum*. A recent study showed that in *M. thermoacetica* the NADH generated during sugar fermentation is converted into the NADPH required for acetogenesis from CO$_2$ by means of an electron-bifurcating NADH-dependent reduced ferredoxin: NADP$^+$ oxidoreductase (NfnAB), a cytoplasmic iron-sulfur-flavoprotein (13).

A different type of energy conservation is found in anaerobic autotrophs harboring the so-called Rnf system. The paradigm of this metabolism is *Acetobacterium woodii*, a nonsporulating bacterium (14). The membrane-bound Rnf complex containing iron-sulfur clusters and flavins was first detected in *Rhodobacter capsulatus* and found to play a role in nitrogen fixation (therefore the designation Rnf, from rhodobacter nitrogen fixation) (15). In *A. woodii*, an Rnf complex was detected and analyzed that coupled electron transfer from reduced ferredoxin to NAD$^+$ to the export of sodium cations (16). Genome sequencing revealed that this is the only Na$^+$ pump in *A. woodii* (17). Thus, the long-standing previous assumption that a methyltransferase, transferring the methyl moiety from methyltetrahydrofolate (THF) to a corrinoid-iron-sulfur protein,

### TABLE 1 Major metabolic features of Clostridium

<table>
<thead>
<tr>
<th>Fermentation pathway</th>
<th>Substrate(s)</th>
<th>Products</th>
<th>Representative species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homoacetate (Wood-Ljungdahl)</td>
<td>Sugars (e.g., fructose), CO$_2$ + H$_2$, CO</td>
<td>Acetate (under special conditions also ethanol)</td>
<td><em>C. aceticum</em>, <em>C. ljungdahlii</em></td>
</tr>
<tr>
<td>Propionate</td>
<td></td>
<td>Propionate, acetate, CO$_2$</td>
<td><em>C. propionicum</em></td>
</tr>
<tr>
<td>Acrylyl-CoA</td>
<td>Lactate</td>
<td>Propionate, CO$_2$</td>
<td><em>C. mayorei</em></td>
</tr>
<tr>
<td>Succinate decarboxylation</td>
<td>Succinate</td>
<td>Butyrate, acetate, CO$_2$, H$_2$</td>
<td><em>C. butyricum</em></td>
</tr>
<tr>
<td>Butyrate</td>
<td>Sugars (e.g., glucose)</td>
<td>Butanol, acetone, CO$_2$, H$_2$, butyrate, acetate</td>
<td><em>C. acetobutylicum</em></td>
</tr>
<tr>
<td>Acetone/butanol</td>
<td>Sugars (e.g., glucose)</td>
<td>Various acids and solvents</td>
<td><em>C. aceticum</em></td>
</tr>
<tr>
<td>Alcohocoltrophic</td>
<td>Various alcohols, alcohol + acid</td>
<td>Various acids</td>
<td><em>C. kluveri</em></td>
</tr>
<tr>
<td>Proteolytic, peptolytic, use of amino acids</td>
<td>Proteins, peptides, amino acids</td>
<td>Acetate, propionate, butyrate, CO$_2$, NH$_4^+$</td>
<td><em>C. propionicum</em>, <em>C. tetanomorphum</em></td>
</tr>
<tr>
<td>Stickland reaction</td>
<td>Pairs of amino acids</td>
<td>Acetate, CO$_2$, NH$_4^+$</td>
<td><em>C. sticklandii</em></td>
</tr>
<tr>
<td>Purinolytic</td>
<td>Purines</td>
<td>Acetate, formate, CO$_2$, NH$_4^+$</td>
<td><em>C. purinilyticum</em></td>
</tr>
<tr>
<td>Pyrimidinolitic</td>
<td>Pyrimidines</td>
<td>Acids, CO$_2$, NH$_4^+$</td>
<td><em>C. oroticum</em></td>
</tr>
</tbody>
</table>
might be membrane bound and, in analogy to methanogens, exploits sodium cations (reviewed in reference 18) turned out to be wrong. Energy conservation is now thought to start with hydrogen oxidation by an electron-bifurcating [FeFe]-hydrogenase, resulting in equal amounts of reduced ferredoxin and NADH. The reduced ferredoxin is oxidized at the Rnf complex, the electrons are transferred to NAD+, yielding NADH, and, during this process, sodium cations are exported. The thus established sodium gradient is used by a Na+-dependent F1F0-ATPase to generate ATP. NADH is used for the reduction of methenyl-THF via methylene-THF to methyl-THF in the methyl branch of the Wood-Ljungdahl pathway. Additional reduced ferredoxin required for CO2 reduction to enzyme-bound CO in the carbonyl branch is thought to stem from the exergonic reduction of methylene-THF to methyl-THF either by electron bifurcation or by direct coupling to the Rnf complex (17). The ATP generated in the acetate kinase reaction is used for the activation of formate to formyl-THF, leaving the sodium cation gradient the only source for ATP formation. A similar mechanism is probably used by C. ljungdahlii, an autotrophic acetogen growing on CO, synthesis gas, and CO2/H2 mixtures, because this bacterium does not harbor genes for cytochromes and quinones, but for an Rnf complex (19). A major difference from A. woodii, however, is that C. ljungdahlii is not sodium dependent. Thus, the coupling ions in this case are probably protons (19). The autotrophic clostridia have gained a lot of industrial interest recently, as they can convert greenhouse gases into valuable bulk chemicals (20, 21). With the use of special media composition, it is also possible to turn, e.g., C. ljungdahlii into an ethanol producer when growing on synthesis gas (20).

**Propionate Fermentation**

The paradigm of a clostridial propionate fermentor is probably *C. propionicum*. It does not use the methylmalonyl-coenzyme A (CoA) pathway, known from propionibacteria, but uses instead the acrylyl-CoA pathway, branched into oxidative and reductive sequences. Lactate is one of the preferred substrates of *C. propionicum*. One molecule is oxidized first to pyruvate and then to acetyl-CoA and CO2. Acetyl-CoA is converted to acetyl phosphate and then to acetate, thereby generating ATP in the acetate kinase reaction. The reducing equivalents obtained in the oxidation steps are used to reduce two molecules of acrylyl-CoA to two propionyl-CoA, which by means of a CoA-transferase are converted into the final product propionate. The two acrylyl-CoAs stem from another two lactate molecules, activated by the CoA-transferase and then dehydrated (3).

Another pathway, i.e., succinate decarboxylation, might be used by *C. mayombei*. This type of metabolism was first discovered in *Propionigenium modestum* (22). Succinate is converted into succinyl-CoA by transfer of the CoA-moiety from propionyl-CoA and subsequent release of propionate. Succinyl-CoA is first rearranged into methylmalonyl-CoA, which is then decarboxylated by a membrane-bound, Na+-exporting decarboxylase into propionyl-CoA and CO2. Propionyl-CoA is used to restart the cycle. The generated sodium cation gradient drives ATP generation by a sodium-dependent F1F0-ATPase. A similar mechanism might be used by *C. mayombei*, because this organism ferments sodium succinate to propionate and CO2 (23). However, this has not yet been experimentally confirmed.

**Butyrate/Butanol Fermentations**

Many clostridia form butyrate as a major fermentation product. The Embden-Meyerhof-Parnas pathway allows degradation of hexoses to pyruvate, which is converted to acetyl-CoA, CO2, and reduced ferredoxin by pyruvate:ferredoxin-oxidoreductase. Part of the acetyl-CoA might be further metabolized to acetate, yielding ATP in the acetate kinase reaction. For butyrate formation, two acetyl-CoAs are combined into acetoacetyl-CoA, which is further converted via 3-hydroxyl-CoA, crotonyl-CoA, and butyryl-CoA into butyrate. Some clostridia use specific phosphotransbutyrylase and butyrate kinase for the last two steps, generating ATP in the kinase reaction. However, the majority use a CoA-transferase and acetate to convert butyryl-CoA into butyrate and acetyl-CoA, the latter of which is then used for acetate and ATP formation (24, 25). However, not only substrate-level phosphorylation during glycolysis and kinase reactions leads to ATP synthesis. Buckel and coworkers provided evidence for a ferredoxin reduction coupled to crotonyl-CoA reduction (26). The butyryl-CoA dehydrogenase/electron transfer flavoprotein (EtfAB) complex catalyzes the exergonic reduction of crotonyl-CoA to butyryl-CoA by NADH and concomitantly drives the exergonic reduction of ferredoxin by another NADH (electron bifurcation). The reduced ferredoxin can then transfer the electrons to NAD+ at the Rnf complex, thus generating an ion gradient, which can be used for ATP synthesis by ATPases.

Not all clostridia do contain an Rnf complex. One of the few exceptions is *C. acetobutylicum*, an organism well known and extensively used for its ability to form...
the solvents butanol and acetone. Butanol is made by the action of butyraldehyde and butanol dehydrogenases from butyryl-CoA, while acetone is formed by decarboxylation of acetoacetate, being derived from acetoacetyl-CoA by a CoA-transferase. In *C. beijerinckii*, acetone can be further reduced to isopropanol. However, all these reactions are only performed at the end of exponential growth, which is fueled by a typical butyrate fermentation. The advantage of switching from an acidogenic to a solventogenic fermentation lies in avoiding life-threatening low pH caused by the acidic fermentation products. As undissociated acids at low pH, they can diffuse across the cytoplasmic membrane and lead to a collapse of the transmembrane proton gradient, as they dissociate in the more alkaline cytoplasm (27). Thus, conversion of acids into solvents allows the cells to stay metabolically active for a longer period and thus gives them an ecological advantage. As solvents become toxic in the long run as well, the induction of sporulation is coupled to the induction of solventogenesis by sharing the same master regulator Spo0A in phosphorylated form (8, 28, 29). For almost one hundred years, the acetone-butanol (AB) fermentation has been a major biotechnological enterprise worldwide. It was second in size to ethanol fermentation. Low crude oil prices and thus cheap chemical synthesis allows the cells to stay metabolically active for a longer period and thus gives them an ecological advantage. As solvents become toxic in the long run as well, the induction of sporulation is coupled to the induction of solventogenesis by sharing the same master regulator Spo0A in phosphorylated form (8, 28, 29). For almost one hundred years, the acetone-butanol (AB) fermentation has been a major biotechnological enterprise worldwide. It was second in size to ethanol fermentation. Low crude oil prices and thus cheap chemical synthesis led to a decline, a trend, which is currently reversed. The use of substrates with no competition to human nutrition, especially, will render the fermentation economically advantageous (30–33).

**Fermentation of Alcohols**

A great variety of mono-, di-, and polyols can be fermented by clostridia. Therefore, only a few examples are provided. Methanol is a substrate of *C. formicoaceticum*; ethanol is a substrate of *C. acetid* (10); ethanol and propanol are substrates of *C. kluyveri*, but only in combination with acetate or succinate (34); butanol is a substrate of *C. ljungdahlii*, but only as a cosubstrate (19); 1,2-ethanediol (ethylene glycol) and 1,2-propanediol are fermented by *C. glycolic* (35); 2,3-butanediol is fermented by *C. acetid* and *C. magnum* (36); and glyceral is fermented by, e.g., *C. aceto*butyricum, *C. beijerinckii*, and *C. butyricum* (37).

The two-substrate fermentation by *C. kluyveri* deserves a more detailed description, because the energetics of this process remained mysterious and were only elucidated a few years ago. Typically, 6 ethanol and 3 acetate (molar ratios are provided) are fermented to 3 butyrate, 1 caproate, and 2 hydrogen. Only 1 ATP is gained in the acetate kinase reaction. It was with this organism that W. Buckel and coworkers discovered the coupling of the exergonic reduction of crotonyl-CoA to butyryl-CoA with NADH by the butyryl-CoA dehydrogenase/EtfAB complex to the endergonic reduction of ferredoxin with NADH (electron bifurcation). Thus, 2 NADH are consumed to generate butyryl-CoA and reduced ferredoxin (38). The reduced ferredoxin can be used for hydrogen formation as well as for additional ATP generation via the Rnf system. Therefore, reduced ferredoxin is oxidized, transferring the electrons to NAD*, and the protons are exported. This proton gradient can then be used by the F1F0-type ATPase. It is assumed that an additional 1.5 ATP are generated this way (39).

**Fermentation of Amino Acids**

Proteins, peptides, and single amino acids are substrates for all proteolytic clostridia. The former two are degraded to monomers by proteases, leaving the amino acids as the starting point for further utilization. Numerous pathways for the various compounds have been elucidated, sometimes even different ones for the same amino acid. Thus, the limited space of this chapter does not allow detailed descriptions of the various degradation sequences. General strategies, however, have been described (40–42). One of the first reactions always is removal of the amino group. This can be achieved by transamination, oxidative deamination, or elimination. In many cases, conversion into the corresponding 2-oxo acids follows. Degradation is often split into oxidative and reductive branches. Typical examples are the utilization of glycine and the degradation of amino acid pairs, the so-called Stickland reaction. Glycine can be used as the sole carbon source by *C. purinilyticum*, an organism specialized on purines and their degradation products (such as glycine) as substrates (43, 44). In the oxidative branch, 1 molecule of glycine is degraded to CO2 via conversion into methylene-THF, with concomitant release of CO2 and NH4*, methenyl-THF, formyl-THF, and finally CO2. The reducing equivalents generated during these reactions are used for reduction of another 3 molecules of glycine to acetate and ammonia. The selenocysteine-containing glycine reductase complex. Acetyl phosphate is then converted into acetate. Thus, from degradation of every glycine molecule, 1 ATP can be formed (via formyl-THF synthetase or acetate kinase, respectively) (44). Similarly, *C. sticklandii* oxidizes 1 molecule of alanine to acetate, CO2, and ammonia to yield sufficient reducing equivalents to convert 2 molecules of glycine to acetate and ammonia via the glycine reductase reaction.
Fermentation of Purines and Pyrimidines

C. acidurici, C. cylindrosporum, and C. purinilyticum are specialized on decomposition of purines. In C. purinilyticum, a selenium-dependent degradation starts by cleaving the pyrimidine moiety of the heteroaromatic ring system. Products are CO₂, NH₄⁺, and imidazole derivatives that are further degraded to formiminoglycine. This compound is cleaved into formimino-THF and glycine, both converted into formate (via methenyl-THF and formyl-THF) and acetate (via glycine reductase and acetate kinase). Thus, from every molecule of formiminoglycine, 2 molecules of ATP can be generated. Depending on the requirement of reducing equivalents, formate can be further oxidized to CO₂ (45). A selenium-independent pathway is possible, but it does not allow good growth. In this case, ring cleavage obviously starts at the imidazole moiety, yielding pyrimidines (46).

Pyrimidine degradation is performed by other specialized clostridia, e.g., C. glycolicum and C. oroticum (47, 48).

Other Fermentations

Clostridia are also able to ferment various organic acids, polymers, halogenated compounds, and aromatic compounds. Some are also able to fix nitrogen (e.g., C. pasteurianum). More details can be found in books completely devoted to the genus Clostridium (1, 2, 49).

SPORULATION IN CLOSTRIDIUM

Morphology of Spores, Spore Contents, and Spore Properties

Like Bacillus, Clostridium species are able to form endospores. Their shape can vary from coccoidal to cylindrical. A series of electron microscopy images taken from sporulating C. formicoaceticum cells resembles, in general, the stages found in Bacillus subtilis (50). Some clostridial spores (e.g., from C. bifermantans) were found to be associated with different appendages, ranging from long, tubular structures to ribbon-like structures or even capped, pin-like protrusions projecting from the spore surface (reviewed in reference 51). Another morphological difference to the archetype of Bacillus sporulation is within Clostridium the formation of so-called clostridial forms at the beginning of sporulation. These cells are swollen, cigar-like, and accumulate storage components such as polysaccharides or polyhydroxybutyrate (Fig. 1). The polysaccharide has been designated granulose and determined in C. saccharobutylicum to consist of an α(1→4)-polyglucan of high molecular weight (52). In C. botulinum, C. butyricum, and C. pasteurianum, similar polymers have been detected, which are, in part, branched and sometimes identical to amylopectin or glycogen (53–57). The existence of this storage material was observed early in the last century, made visible by iodine staining, and used for differentiation (58, 59). Clostridia also produce dipicolinic acid, which complexes Ca²⁺ ions and thus adds to the thermoresistance of spores. However, several species (including C. acetobutyllicum, C. beijerinckii, C. botulinum, C. perfringens, and C. tetani) lack the genes spoVFA/B, whose products catalyze the oxidation of 2,3-dihydrodipicolinic acid (DHDPA) (an early intermediate in bacterial lysine biosynthesis) to dipicolinic acid in Bacillus species. In C. perfringens, the EtfA protein produces dipicolinic acid from DHDPA (60), indicating an additional role for this protein to the one in electron bifurcation during the reduction of crotonyl-CoA to butyryl-CoA. In Bacillus, small, acid-soluble spore proteins (SASPs) exert a protective effect against heat, peroxides, as well as UV radiation and also change the DNA conformation into an A-helix. Such proteins have also been detected in C. bifermantans and C. perfringens (61, 62) and since then in the sequenced genomes of clostridia. SASPs are classified into α/β and γ types, of which Clostridium, in contrast to Bacillus, obviously does not contain genes encoding γ-type SASPs (63). In C. perfringens, analyses of respective mutants and by antisense RNA showed that α/β-SASPs contribute significantly to spore resistance against moist heat, UV radiation, hydrogen peroxide, hydrochloric acid, nitrous acid, and formaldehyde (64–66).

Because bacterial endospores represent the most resistant cell type known (highly resistant to heat, chemicals, desiccation, and radiation), the presence of spores of pathogenic clostridia in food would be a serious problem. Therefore, measures are taken to prevent contamination in the first place (hygienic conditions, vacuum packing). Although the shelf life of food can be increased by a number of treatments, this does not per se result in the inactivation of spores. Rather, it prevents germination. Thus, the addition of preservatives such as nitrite has proved to be useful. Nitrite destroys redox-active iron-sulfur clusters, which are parts of essential

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enzymes in the vegetative cells of clostridia (67, 68). Not only do the spores of the well-known pathogens such as *C. botulinum*, *C. difficile*, *C. perfringens*, and *C. tetani* represent a threat, but also the spores of species that spoil food so that it can no longer be sold and used. Such an example is *C. tyrobutyricum* in the dairy industry. Because the organism converts lactate and acetate into butyrate and CO₂, it outcompetes the *Propionibacterium* used in cheese making and changes the taste and structure of the product (so-called late blowing).

Spores might also exhibit some metabolic activities without starting to germinate. In the spore coat of a number of *Firmicutes*, among them *C. tetani* and *C. thermocellum*, a new family of bacterial spore kinases has been detected; their physiological function is still unknown (69). *C. acetobutylicum* spores are obviously able to reduce uranyl acetate (U(VI)) to a mixture of UO₂ and other U(IV) products, when hydrogen is provided as an electron donor. The U(IV) products form a precipitate around the spores (70). Purified spores of *C. difficile* have been shown to exhibit catalase, peroxiredoxin, and chitinase activity, catalyzed by CotCB, CotD, and CotE, respectively. CotE is bifunctional, the latter two enzymatic activities are located at the N-terminal and C-terminal domains, respectively (71).

**Mechanism of Sporulation**

In *B. subtilis*, sporulation is initiated by phosphorylation of Spo0F, the first component of the so-called phosphorelay, by one of five different interacting histidine kinases. The His to Asp phosphotransfer is as in a classical two-component system and is repeated with the following proteins. Spo0B accepts the phosphoryl group from Spo0F∼P at a histidine residue and then transfers it to Spo0A, again to an aspartate residue. Spo0A∼P then becomes the master regulator of sporulation. This phosphorelay allows the integration of signals not only at Spo0F, but also at Spo0B or Spo0A. So, it came somewhat as a surprise when genome sequencing revealed that no *spo0F* and *spo0B* genes were found within the genus *Clostridium* (72–74). Thus, clostridia do not possess a phosphorelay. Considering the evolutionary relationship between *Clostridium* and *Bacillus* (clostridia appeared as a separate class approximately 2.7 billion years ago, bacilli approximately 2.3 billion years ago), this makes sense, because the adaptation to an aerobic lifestyle required enhanced sensing and regulating capabilities, which probably resulted in development of the phosphorelay (75, 76). On the other hand, Spo0A is indeed the master regulator of sporulation, and the respective gene has been found in all endospore formers (77). Studies on the molecular evolutionary history of this protein in all respective
genera have revealed a division into two clades, mostly representing *Bacillus* and *Clostridium* species (78).

Phosphorylated Spo0A initiates sporulation in *Clostridium* as in *Bacillus* (79). In concert with σH, a number of operons are transcribed. Then, in the forespore, σF becomes active, when the anti-sigma factor SpoIIAB (ADP form) binds to the anti-anti-sigma factor SpoIIAA in its unphosphorylated form. The phosphorylated form of SpoIIAA, found predominantly in the mother cell, binds σF and thus prevents its transcriptional activity. SpoIIAB (ATP form) is responsible for both, the binding of σF and the phosphorylation of SpoIIAA, while SpoIIE catalyzes dephosphorylation. σ-dependent transcription leads to the formation of SpoIIR, which interacts with the membrane-bound protease SpoIIGA. SpoIIGA is responsible for the cleavage of pro-σF in the mother cell, yielding active σF. This process is supported by higher concentrations of Spo0A~P in the mother cell (as a consequence, higher concentrations of pro-σF in the mother cell) and a degradation of pro-σF in the forespore. After σF and σE become specifically active in the forespore or mother cell, respectively, the mother cell engulfs the forespore. Then, a second set of sigma factors enters the stage: σG in the forespore and σK in the mother cell. The anti-sigma factor SpoIIAB also acts on σG in the forespore, but additional components are required for activation (including SpoIIIJ, triggered by σE-dependent SpoIIAA from the mother cell). σK in the mother cell is first synthesized as an inactive precursor (similar to σF) and is activated by proteolysis. Again, help from the forespore is required for this process in the form of SpoIVB. The *spoIVB* gene is under control of the forespore-specific sigma factor G, and the protein is assumed to be inserted into the inner forespore membrane and to undergo autoproteolysis. Thus, released fragments serve as signals and interact with the SpoIYFA/FB-BofA complex in the outer forespore membrane. All these proteins stem from σE-dependent expression in the mother cell. The complex, upon interacting with fragments of SpoIVB, then cleaves pro-σK, yielding active σK. σG and σK allow expression of those operons, whose products are required for spore maturation. This whole process seems to be identical to what is happening in *C. acetobutylicum* during sporulation, the best-studied *Clostridium* in this respect. Genes encoding σE, σF, σG, and σK have been identified (80, 81), and the time course of expression of σF, σG, and σK as judged by Northern blots followed the scheme identified for *Bacillus* (82). However, closer inspection of regulons and their expression has revealed several differences. With the use of the *C. acetobutylicum* sigF mutant and complemented strain, it was found that σF was the first sporulation-related sigma factor to become active and that it is needed for the expression of σE and σG (83). This is in line with the *Bacillus* model and also true for *C. perfringens* (84). However, while sigG expression was found to be σE-dependent, this was not the case for the genes *gpr, lonB, spoIIB*, and *spoIIR*, clearly a difference from *B. subtilis* (83). Reporter gene analyses led to the conclusion that SpoIIE controls sporulation in a manner similar to that in *Bacillus* (85), while investigations with a *spoIIE* mutant of *C. acetobutylicum* showed that SpoIIE affected sigF transcription and indicated an autostimulatory role for σF (86). A sigE mutant of *C. acetobutylicum* seems to be stalled in sporulation before stage II (87), which is in contrast to *B. subtilis* and also *C. perfringens* sigE mutants, stalled at early stage II (88). σE activity in *C. acetobutylicum* is also required for granulose synthesis and morphology of the "clostridial form." A *C. acetobutylicum* sigG mutant seemed to advance further in sporulation than a respective *Bacillus* mutant (87). The most important difference, however, seems to be the role of σK. A genome comparison of *Bacillus* with various clostridia has already revealed a lack of conserved genes beyond the developmental stage of *spoIVB* (78). This was followed by a detailed transcriptional study in *C. acetobutylicum*, providing the most comprehensive roadmap of clostridial sporulation up to now (89). The gene, originally identified as sigK (81), did not show increased expression, and the transcript level was well below those of the other sigma factor genes. Expression of two genes, known to be σK controlled in *Bacillus*, was also tested, but the data did not allow an unambiguous decision of whether there is a functional σK or not (89). On the other hand, the study confirmed the sequential order of σH, Spo0A, σF, σE, and σG in *C. acetobutylicum*, thus being identical to the scheme known for *Bacillus*. Genome sequencing revealed that sigK genes in most clostridia are intact as in most bacilli. *C. tetani* resembles *B. subtilis* by carrying a skin (sigK intervening sequence) element within the sigK gene. In *C. difficile*, a smaller skin element with divergent orientation was detected. In vivo excision of this element was found to be essential for sporulation (90). In *C. botulinum*, a sigK gene was disrupted (91). As a consequence, sporulation was blocked at an early stage, rather than at late stage as is the case in *Bacillus*. Thus, the function of σK (or a similar sigma factor) might be somewhat different in clostridia. In this respect, it has also been shown that σH1 in *C. difficile* governs the expression of many more genes than its counterpart in *Bacillus* (92).
Sporulation frequency in clostridia is not type associated, but rather is strain dependent, as shown for *C. difficile* strains (93, 94).

Regulation of Sporulation

The first experiments that indicated the close relationship of *Bacillus* and *Clostridium* at the molecular level were the discovery of the common presence of Spo0A in both genera and the detection of sporulation-specific sigma factor homologs in *C. acetobutylicum* (77, 81). Since then, it has been an open question concerning how Spo0A becomes phosphorylated to start the sporulation process. A comparative genomic study revealed a number of orphan histidine kinases in *C. acetobutylicum, C. perfringens*, and *C. tetani* (95). A transcriptional expression profile further narrowed down the number of potential candidates (75). Recent experiments based on newly developed mutation techniques for *Clostridium* finally provided compelling evidence for the situation in *C. acetobutylicum* (96). By insertional inactivation of all histidine kinase candidates (single and double mutants), Young and his coworkers could show that two different pathways exist for Spo0A phosphorylation in *C. acetobutylicum* (Fig. 2). One way of signal transduction occurred by concerted action of the kinases Cac0903 and Cac3319. Cac0903 showed the dominant activity at the transcript level. In addition, spo0A expression was found to be Cac0903 dependent, indicating that Spo0A~P induces spo0A expression as also found in *Bacillus*. The other pathway was governed by Cac0323 kinase alone. A fourth kinase, Cac0437, probably acted as a phosphatase of Spo0A~P, thus fulfilling the roles that Spo0E, YnzD, and YisI play in *Bacillus*. Interestingly, this reaction was ATP dependent and its mechanism still awaits elucidation. Steiner et al. (96) pointed out some similarity to the *Myxococcus xanthus* RedCDEF system, in which RedE becomes phosphorylated and then dephosphorylates RedF~P. However, there might also be the possibility of different sensor kinases forming heterodimers, leading to a different signal output (97).

The kinases found to be interaction partners of Spo0A in *C. acetobutylicum* must not be the same as in other clostridia. Genome comparisons revealed that an
ortholog of Cac3319 is present in C. tetani, but not in C. perfringens. Similarly, orthologs of Cac0323 and Cac0903 can be found in C. perfringens, but not in C. tetani (97). This somewhat reflects the situation in Bacillus. While B. subtilis possesses 5 kinases that are able to phosphorylate Spo0F, Bacillus anthracis contains 8 and Bacillus cereus even 11 (76). A reason might be that the bacteria use Spo0A to control additional networks to sporulation. In C. acetobutylicum, Spo0A–P also serves as the master regulator of solvents production, being responsible for the onset of acetone and butanol production (28, 29, 31, 98–100). In C. perfringens, production of the enterotoxin (CPE) is coupled to sporulation. CPE formation is \( \sigma^E \) and \( \sigma^K \) dependent (88). Another C. perfringens toxin, TpeL, belongs to the family of large clostridial cystotoxins, which use UDP-glucose as a substrate and glycosylate G-proteins such as Rac, Ras, and Rho (with some diversity among different toxins with respect to target proteins). TpeL is also expressed during sporulation, being controlled by Spo0A and \( \sigma^E \) (101). The well-known members of the same toxin family, toxin A and toxin B of C. difficile (encoded by tcdA and tcdB, respectively), are also massively produced during the stationary growth phase (102). They are under control of the alternative sigma factor TcdR and the anti-sigma factor TcdC (103). A link to sporulation obviously exists, but the data reported are somewhat contradictory. Using the new mutation techniques already mentioned, the spo0A gene and the gene of one of the orphan kinase interaction candidates were insertionally inactivated (104). The loss of Spo0A abolished sporulation completely as expected. In parallel, the concentration of toxin A, both intracellularly as well as extracellularly, was dramatically reduced. This was also the case, however, to a much lower extent, with the CD2492 kinase mutant. The latter result would be consistent with this kinase just being one of several that interact with Spo0A. However, it must be kept in mind that no Spo0A binding sites (so-called 0A boxes) have been found upstream of tcdA and tcdB (104). So, an indirect link must be assumed. On the other hand, a recent publication confirmed an influence of Spo0A on toxin production, but in a negative way. Again, a spo0A mutant of C. difficile was used. In this study, inactivation of spo0A led to increased production of toxins A and B (105). A possible reason might be the use of different strains, as was probably the case with two studies reporting conflicting data that only toxin B is essential for virulence of C. difficile (106) versus cytotoxic activity and corresponding virulence exerted by single action of toxin A as well as toxin B (107). In C. botulinum, production of the binary toxin C2 is linked to sporulation (108); however, the molecular details are not yet known.

A recent finding is that sporulation in pathogenic as well as apathogenic clostridia is regulated via the agr quorum-sensing system of Gram-positive bacteria. The membrane-associated AgrB protein processes and exports the autoinducer peptide, which is encoded by agrD. Sensing and regulation is achieved by the sensor kinase AgrC and the response regulator AgrA. An agrD mutant of C. botulinum showed a drastically reduced sporulation frequency and also reduced formation of neurotoxin. In C. sporogenes, AgrB activity was reduced by an antisense-RNA approach, which also led to an approximately 100-fold reduced sporulation frequency (109). An agrB mutant of C. perfringens could no longer produce spores and was blocked in enterotoxin (CPE) and beta2 toxin formation. Synthesis of alpha toxin and perfringolysin O was also reduced. Western blots revealed reduced levels of Spo0A as well as \( \sigma^E \) and the absence of \( \sigma^G \) in the mutant (110). In C. acetobutylicum, agrA, agrB, and agrC mutants could be constructed. All mutants no longer produced granulose. On solidified media, agrA and agrC mutants showed a significantly reduced spore formation, 5 orders of magnitude lower than the wild type. Solvent formation was not affected in all mutants. Mutations could be restored by adding culture supernatant or synthetic autoinducer peptide (111). The agr system is obviously present in all clostridia as judged from genome data. As the response regulator AgrA controls directly a number of operons and also induces expression of the small, noncoding RNA RNAIII, it will be interesting to see whether such a sRNA is involved in control of sporulation. There is a recent report on CsG, a sporulation-specific, small, noncoding RNA, which is highly conserved in endospore formers (112). It is synthesized exclusively in the forespore, but its physiological function seems to be in supporting later germination rather than the onset of sporulation (112).

**Germination of Clostridial Spores**

Under appropriate environmental conditions, a spore can germinate and thus convert back into a fully active vegetative cell. This can be achieved by inducing compounds, designated germinants, and physical factors. Interaction of germinants with specific receptors in the inner spore membrane first stimulate the release of H^+, K^+, and Na^+. Then, dipicolinate and Ca^{2+} ions as well as other divalent cations are set free, accompanied by imbibition of water. Potassium ions are reabsorbed...
by specific transporters. Hydrolysis of the spore’s peptidoglycan cortex by spore cortex lytic enzymes (SCLEs) allow further uptake of water and swelling of the cell. SASPs are degraded by germination proteases; the resulting amino acids are then used for protein synthesis and energy metabolism.

L-Alanine has been identified as a germinant for *C. botulinum* and *C. sporogenes* spores (113); l-asparagine, a mixture of KCl and l-asparagine, l-alanine, and l-valine, and Na*+* ions together with inorganic phosphate, all to various degrees, for *C. perfringens* spores (114, 115); and inorganic phosphate, bile salts, and glycine for *C. difficile* spores, although with substantial diversity among different strains (116–118).

Germinant receptors belong to the GerA family and are organized in *B. subtilis* in three tricistronic operons (gerA, gerB, and gerK). Organization in clostridia is somewhat different and also strain dependent. The products of the *gerK* operons of *C. perfringens*, organized in a monocistronic *gerKB* and a bicistronic *gerKA/C* transcriptional unit, were found to be essential for l-asparagine-dependent germination and also to participate in KCl-induced germination (114). GerKA and GerKC seem to be the major players, because GerKB has an auxiliary role in spore germination, but is required for spore viability and outgrowth (119). On the other hand, GerA, being the product of the monocistronic *gerA* operon, obviously had only little effect on germination, as determined by mutant analyses (114).

Ion transport during *C. perfringens* spore germination might in part be catalyzed by GerO, a putative antiporter for Na*/H*/K*. This protein was found to be essential for germination, but might also play a role in spore formation (120). In *C. perfringens*, there are different spore-cortex lytic enzymes: an N-acetylmuramyl-l-alanine amidase, acting on the intact spore cortex and designated SCLE (encoding gene is *sleC*), and an N-acetylmuramidase, acting on the disrupted peptidoglycan fragments and designated CFLE (cortical fragment-lytic enzyme, encoding gene is *sleM*) (121). SleM and SleC have been localized on the outside of *C. perfringens* spore cortex (122). SleC probably also exerts transglycosylase activity (123) and was found to be essential for germination of *C. perfringens* spores, in contrast to SleM (124). SleC is also present in *C. difficile* and also essential for germination (125). A genome comparison between 12 *Bacillus* and 24 *Clostridium* strains revealed two major groups of germination-specific lytic enzymes in *Clostridium* (126). *C. beijerinckii* and *C. botulinum* Eklund 178 carried genes with homology to *C. perfringens* *sleC*, *sleM*, *gpr* (encoding a germination-specific protease degrading SASPs), *C. acetobutylicum*, *C. difficile*, and *C. botulinum* Alaska E43 homologs of *sleC* and *gpr*. These species and strains thus group with *C. perfringens*. On the other hand, *C. cellulolyticum*, *C. kluyveri*, *C. novyi*, *C. tetani*, and most *C. botulinum* strains carry genes with homology to *Bacillus subtilis*, *sleB*, and *ypeB*.

**Industrial and Medical Applications of Clostridial Spores**

An industrial application for clostridial spores is the use of *C. butyricum* spores as a probiotic (127). A general advantage of sporeformers as probiotics is that the number of spores added to food can be reduced by about 100-fold in comparison with vegetative cells. *C. butyricum* strain Miyairi 588 spore preparations have been commercially available since 1968 in Japan, China, and Korea and are used as a *C. difficile* prophylaxis and as treatment against non-antimicrobial-induced as well as antimicrobial-associated diarrhea. Spore preparations are produced by Miyarisan Pharmaceutical Co. Ltd., Tokyo, Japan. In 2012, approval as a novel food supplement within the European Union was requested (Miya-Pro tablets, [http://www.food.gov.uk/multimedia](http://www.food.gov.uk/multimedia). These tablets contain a concentrate of *C. butyricum* Miyairi 588 spores. The history of the strain dates back to 1933, when it was isolated by C. Miyairi. The 588 strain (the 588th isolate) was isolated in 1963. *C. butyricum* Miyairi 588 was shown to have therapeutic efficiency against inflammatory bowel disease because of its butyrate formation (128) and, as an agonist, to have preventive and therapeutic effects on a number of pathogens, among them *C. difficile* and enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 infection in a gnotobiotic mouse model (129).

An important medical application is the use of recombinant clostridial spores for cancer treatment (130–132). Because clostridia are anaerobic bacteria, their spores will only germinate in anaerobic environments. Normally, the tissues of humans and mammals in general are well oxygenated. Only within a fast-growing tumor and its surroundings does oxygen become limited and the tissue thus hypoxic. Thus, in healthy mammals, clostridial spores are removed quickly and without any deleterious effects (133, 134). However, when carrying a tumor, the mammal is colonized at and in the tumor. Clostridial spores recognize the hypoxic conditions, germinate, and the vegetative cells proliferate at the expense of the necrotic tissue. Thus, targeting of the tumor by
clostridial spores is very specific, and the multiplication of bacterial cells at the target takes place. If the clostridia are now engineered to carry a gene whose gene product is toxic against tumor cells, a perfect and safe delivery vehicle has been developed. Safety is guaranteed because antibiotic treatment can kill and remove the microbes at any time. Three principal strategies are currently being tested: (i) the introduction into Clostridium of a gene whose gene product acts directly against the tumor (e.g., tumor necrosis factor, interleukin-2, C. perfringens enterotoxin) (3, 133, 136); (ii) introduction into Clostridium of a gene whose gene product converts an innocuous prodrug, injected separately after successful colonization, into a cytotoxic drug (e.g., cytokine deaminase and 5-fluorocytosine/5-fluorouracil); other enzymes are carboxypeptidase and nitroreductase) (reviewed in reference 132); and (iii) intravenous injection of nontoxic C. novyi together with conventional chemotherapeutic drugs (137). An important future step will be to seek and win FDA approval for respective clinical studies.

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