

Diphtheria Disease and Genes Involved in Formation of Diphthamide, Key Effector of the Diphtheria Toxin

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1. Introduction

Within shared ecological niches, secretion of lethal protein toxins by microorganisms is a common strategy to ensure positive selection and survival of the toxin producing killer strains amongst other microbial competitors including bacteria, yeast and fungi (Schmitt & Schaffrath, 2005). Frequently, toxin production and secretion are traits that are genetically associated with extrachromosomal elements such as linear DNA killer plasmids and double-stranded RNA mycoviruses from yeast and fungi or episomal and prophage DNA integrated into the chromosome of the microbial toxin producer (reviewed by Meinhardt et al., 1997; Schaffrath & Meinhardt, 2005; Leis et al., 2005). The latter scenario is predominantly found in bacterial species and accounts for lysogenic conversions, phenomena in which phenotypes of the microbial host including pathogenicity, growth performance and production of virulence factors and toxins can be significantly affected by expression of prophage encoded genes. Prominent examples for phage-dependent toxin expression and disease formation include exotoxin-associated scarlet fever by *Streptococcus pyogenes* (Johnson et al., 1986; Broudy et al., 2001), dysentery causing Shiga toxins Stx1 and Stx2 from *Shigella dysenteriae* (Newland et al., 1985; Willshaw et al., 1985; Huang, et al., 1986), phage CTX ϕ encoded cholera toxin from *Vibrio cholera* (Waldor & Mekalanos, 1996; Faruque & Nair, 2002) and diphtheria toxin encoded on a beta prophage from lysogens of *Corynebacterium diphtheriae* (Holmes & Barksdale, 1969; Bishai & Murphy 1988). Together

with tRNase ribotoxins and anticodon nucleases from prokaryal and eukaryal microbes that have been shown to be encoded by transposable elements as well as circular and non-conventional linear DNA plasmids (Tokunaga et al., 1990; Kaufmann, 2000; Schaffrath & Meinhardt, 2005; Schaffrath et al., 1999), all of these genetic constellations implicate scenarios in which killer phenotypes have been evolved and spread by way of viral DNA transduction pathways or other forms of horizontal gene transfer. In support of this notion, certain cytoplasmic killer plasmids and their associated toxin phenotypes can be transferred between distinct yeast genera by means of cytoduction (Gunge 1983; Sugisaki et al., 1985) and horizontal transfer of the diphtheria toxin encoding *tox* gene from phages has been assigned to *in situ* lysogenic conversion of non-toxigenic to virulent corynebacteria (Freeman, 1951).

With regards to individual toxin response pathways in sensitive target cells, the specific cellular components being targeted by individual microbial protein toxins, not surprisingly, vary significantly depending on the nature of the essential cellular process that is targeted by the toxin in question (Schmitt & Schaffrath, 2005). For instance, microbial toxins capable of inhibiting the process of protein biosynthesis not only have been shown to target individual steps of mRNA translation (e.g. initiation, elongation and termination) but also to attack different components of the ribosomal machinery or associated factors required for mRNA translation (e.g. proteins, mRNAs, tRNAs and rRNAs). In this review, we will focus on one such microbial protein toxin that targets the essential process of mRNA translation and protein biosynthesis: diphtheria toxin (DT) from the Gram-positive bacterium *Corynebacterium diphtheriae* (for previous reviews, see Pappenheimer, 1977, 1984; Murphy, 1996). For DT to unfold its lethal action, the toxin needs to hijack a post-translationally modified residue known as diphthamide in its target protein EF2 (eukaryotic translation elongation factor 2). Next and by virtue of its enzymatic activity, DT ADP-ribosylates its target protein, an irreversible modification that inactivates the essential function of EF2 in mRNA translation. Eventually, EF2 inactivation by DT causes depletion of *de novo* protein biosynthesis and results in the death of the target cell including the model eukaryote and budding yeast *Saccharomyces cerevisiae* (reviewed by Collier, 2001; Todar 2004; Ratts & Murphy, 2005). Here, we review recent advances in the molecular biology of DT and present new insights into DT mode of action and DT response pathway components. An attractive idea emerging from research into DT mode of action is to take its basic molecular biology and apply it to biomedical intervention schemes against tumour cells, microbial pathogens or other biomedically and biotechnically relevant cell systems whose proliferation heavily relies on mRNA translation and protein biosynthesis (White-Gilbertson et al., 2009; Uthman et al., 2011). Such strategies are particularly informed by the use of chimeric DT fusion proteins that combine the lethal ADP-ribosylation activity of DT with a specific cell surface receptor domain for target cell or tissue specificity (Kreitman, 2006, 2009).

2. History and discovery of the diphtheria pathogen

Since the discovery in 1884 by German bacteriologists and physicists Edwin Klebs (1834–1912) and Friedrich Löffler (1852–1915), that *Corynebacterium diphtheriae* (also known as the *Klebs-Löffler* bacillus) is the causative agent of diphtheria (Fig. 1), diphtheria has arguably developed into one of the prototypic, toxigenic and infectious human diseases. Soon after Löffler speculated that organ damage during diphtheria was the consequence of a bacterial

toxin, French Pierre-Paul-Émile Roux (1853-1933) and Swiss-French Alexandre Émile Jean Yersin (1863-1943) showed elegantly at the Pasteur Institute that the major bacterial virulence factor was indeed a potent exotoxin (Roux & Yersin, 1888). Upon sterile-filtration of *C. diphtheriae* cultures, they injected toxin-containing, cell free supernatants into laboratory animals and found that disease symptoms (including the eventual death of the animals) were developed in a manner indistinguishable from animals infected with the bacterium alone or even from infected humans. In addition, they proved that toxin-containing urine obtained from children infected with *C. diphtheriae* was sufficient to induce the disease symptoms seen in the above laboratory animals.

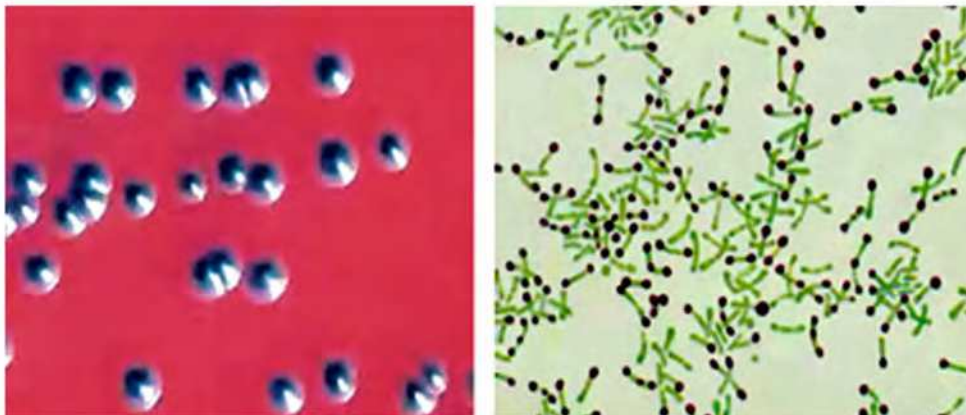


Fig. 1. *Corynebacterium diphtheriae*, the causative agent of the diphtheria disease. Formation of *C. diphtheriae* colonies is shown on blood agar (left panel) according to the CDC (Centers for Disease Control and Prevention, USA). Stained cells of *C. diphtheriae* (right panel). Their barred appearance is due to metachromatic granules which contain polyphosphate. Permission by Professor Kenneth Todar, University of Wisconsin, USA, to show the photographs (Todar, 2004) is gratefully acknowledged by the authors.

Löffler was the first to show that the pathogen could selectively be cultured from nasopharyngeal infections, indicating that diphtheria spreads within the upper respiratory tract. The disease causes a sore throat, low fever and an adherent green-grey membrane on the tonsils, pharynx, and nasal cavities. This thick and fibrinous pseudomembrane, which can severely obstruct airways and suffocate patients, is the result of a combination of bacterial and host effects in response to pathogen cell growth and toxin production as well as the host's immune response and necrosis of the underlying host cell tissue. In 1890, German scientists Emil Adolf von Behring (1854-1917) and Paul Ehrlich (1854-1915) began to study the immunization of horses against diphtheria in order to generate a serum for medical use in humans. Considered to be Ehrlich's first bacteriological achievement attracting world-wide renown, the transformation of diphtheria antitoxin into an effective protective preparation was successfully used during an epidemic in Germany. Rather controversially, however, only von Behring was awarded the first Nobel Prize in Medicine in 1901 for developing a serum therapy against diphtheria.

3. Manifestations, pathogenesis and epidemiology of diphtheria

There are two disease forms, cutaneous and nasopharyngeal diphtheria (Fig. 2). The latter may vary from mild pharyngitis to hypoxia and suffocation with symptoms including fever of more than 39.5°C (~103°F) and profound swelling of the neck upon cervical lymph node infections (also known as bull neck diphtheria). Ultimately, diphtheria may cause life-threatening complications including loss of motor function and difficulty in swallowing and/or congestive heart failure as a result of diphtheria toxin (DT) induced myocarditis (~20% of cases) and peripheral motor neuropathy (~10% of cases) (Fig. 2) (Solders et al., 1989; Havaladar et al., 2000). Diphtheritic skin lesions in the milder form of cutaneous diphtheria are also covered by the typical pseudomembrane (see above). Eventually, DT distribution by way of the circulatory system may reach distant organs and cause paralysis (Fig. 2). Asymptomatic nasopharyngeal carriage is common in regions where diphtheria is endemic. In susceptible individuals, toxigenic strains cause disease by multiplying and secreting DT in either nasopharyngeal or skin lesions (Fig. 2). The diphtheritic lesion is often covered by the pseudomembrane which is composed of fibrin, bacteria, and inflammatory cells (Fig. 2).

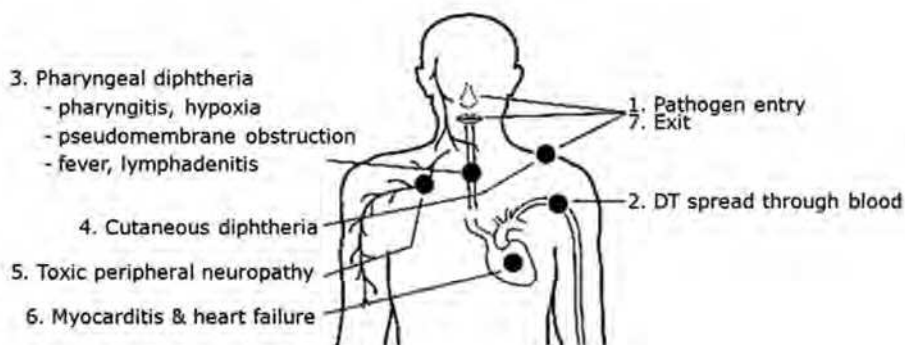


Fig. 2. Pathogenesis of the diphtheria disease. The illustration summarizes clinical manifestations of both pharyngeal and cutaneous diphtheria as well as the spread of the disease which involves blood-borne diphtheria toxin (DT) rather than distribution and dissemination of the pathogen *C. diphtheriae*. Systemic complications are indicated by the occurrence of toxic peripheral neurotrophly and toxic myocarditis eventually causing congestive heart failure. The scheme represents a modified version inspired by a comprehensive diphtheria review (Murphy, 1996)

Diphtheria pathogenesis is largely determined by the capacity of virulent *C. diphtheriae* biotypes (see below) to produce the deadly DT and to colonize and multiply in skin lesions or nasopharyngeal cavities. Since both determinants are encoded by the bacterium carrying a lysogenic beta prophage (Freeman, 1951; Bishai & Murphy, 1988), virulence results from the combined effects of the bacterial and phage genomes (Fig. 3). Even though avirulent *C. diphtheriae* strains seldom associate with the disease, it has been documented that non-

toxigenic strains may acquire virulence following lysogenic conversion *in situ* (see below). Although it is clear that events other than production and secretion of DT promote host tissue colonization, detailed knowledge about *C. diphtheriae* factors involved in virulence is scarce. Putative roles in the colonization process have been discussed for a sialic acid splitting neuraminidase from the pathogen and for a corynebacterial cell surface component known as cord factor, i.e. 6,6'-di-O-mycoloyl- α , α -D-trehalose (reviewed by Murphy, 1996). The emergence of continuously changing lysotypes in the pathogen's population is likely due to their ability to compete more efficiently in segments of the nasopharyngeal ecologic niche. Thus, a given lysotype may persist for a while only to be replaced during later stages in the infection by another one that is more adapted to its niche.

Diphtheria is a contagious disease; although toxigenic strains have been isolated from horses, the pathogen *C. diphtheriae* is usually spread by direct physical contact among humans, namely by droplets or through inhaling aerosols of infected individuals. In addition, *in situ* conversion of avirulent strains to pathogenic ones may involve lateral gene transfer following bacterial lysis and release of the DT encoding phage gene (Freeman, 1951; Holmes & Barksdale, 1969). In regions of active immunization programs, isolated focal outbreaks can be associated with carriers who returned from visits to regions still endemic for diphtheria. In the US, Europe and elsewhere, diphtheria was a disease typical of children before mass immunization with diphtheria toxoid. Today, virulent biotypes of *C. diphtheriae* are rarely isolated and clinical diphtheria has largely been eradicated from industrialized nations through global vaccination schemes. Due to effective Diphtheria-Pertussis-Tetanus (DPT) vaccines, the number of diphtheria cases among school-aged children in the US has significantly dropped from 52 in 1980-2000 to 3 in 2000-2007 (Atkinson et al., 2007). For adults, however, vaccine boosts, are strongly advocated since immunity wears off with age and 30-60% of adults are estimated to be at risk, in particular, persons travelling to countries in which diphtheria has not been fully eradicated but poses a constant endemic health-threat. In the 1980s and 1994, public health breakdown in Sweden and Russia caused epidemic clinical diphtheria (Rappuoli et al., 1988) with officials recording more than 80,000 cases including 2,000 deaths. Focal outbreaks reported thereafter were almost certainly associated with diphtheria carriers who returned from Russia to Europe and the US.

4. Host defence, diagnosis and control

Recognition that systemic organ damage associated with diphtheria is due to the action of the lethal DT rather than dissemination of blood-borne pathogens led to the development of a highly successful toxoid vaccine in which inactivated DT that remains antigenic is able to raise an immune response. The toxoid is prepared by incubating DT with formaldehyde at 37° C under alkaline conditions. Although immunization with the toxoid has made diphtheria a rare disease, diphtheria outbreaks do still occur in non-immunized and immune-compromised groups. Control of diphtheria, therefore, depends upon adequate immunization with antigenically intact yet inactivated diphtheria toxoid. Immunization against diphtheria should begin in the second month of life with a series of three primary doses spaced 4 to 8 weeks apart, followed by a fourth dose approximately 1 year after the last primary inoculation. Diphtheria toxoid is widely used as a component in the DPT vaccine (see above). Epidemiologic surveys have shown that immunization against diphtheria is approximately 97% effective. Although mass immunization against diphtheria

is practiced in the United States and Europe and there is an adequate immunization rate in children, a large proportion of the adult population may have antibody titers that are below the protective level. The adult population should be reimmunized with diphtheria toxoid every 10 years (see above). Indeed, booster immunization with diphtheria-tetanus toxoids should be administered to persons traveling to regions with high rates of endemic diphtheria (Central and South America, Africa, Asia, Russia and Eastern Europe). In recent years, the use of highly purified toxoid preparations for immunization has minimized the occasional severe hypersensitivity reaction.

Although antibiotics (e.g. penicillin and erythromycin) are used as part of the treatment of patients who present with diphtheria, prompt passive immunization with diphtherial antitoxin is most effective in reducing the fatality rate. The long half-life of specific antitoxin in the circulation is an important factor in ensuring effective neutralization of diphtheria toxin; however, to be effective, the antitoxin must react with the toxin before it becomes internalized into the cell. Protection and immunity towards diphtheria involves an antibody response to DT following clinical disease or a formaldehyde-inactivated diphtheria toxoid following immunization. Immunization with diphtheria toxoid is extraordinarily effective. Diphtheria patients must be promptly treated with antitoxin to neutralize circulating DT.

Clinical diagnosis of diphtheria requires bacteriologic laboratory confirmation of toxigenic *C. diphtheriae* in throat or lesion cultures. For primary isolation, a variety of media may be used including Löffler, Müller-Miller tellurite or Tinsdale tellurite agars. Sterile cotton-tipped applicators are used to swab the pharyngeal tonsils or their beds. Calcium alginate swabs may be inserted through both nares to collect nasopharyngeal samples for culture. Since diphtheritic lesions are often covered with a pseudomembrane (see above) the surface of the lesion may have to be carefully exposed before swabbing with the applicator. In addition to the determination of biotype and lysotype of *C. diphtheriae* isolates, it is possible to use molecular biology techniques in the study of diphtheria outbreaks and pathogen identification. The latter may involve restriction endonuclease digestion patterns of *C. diphtheriae* chromosomal DNA as well as the use of the cloned DT gene (*tox*; see below) or specific corynebacterial insertion sequences as genetic probes (von Hunolstein et al., 2003).

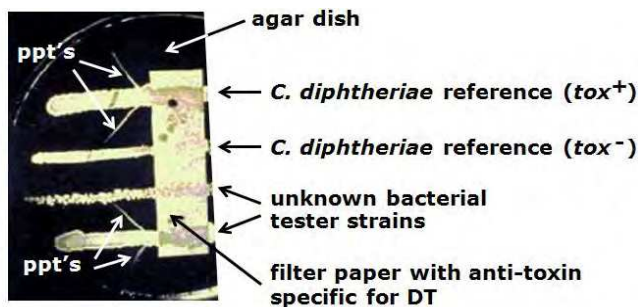


Fig. 3. The Elek immunodiffusion test. Shown is an agar plate with an antitoxin soaked filter that is inoculated with known toxigenic (tox^+) and non-toxigenic (tox^-) isolates of *C. diphtheriae* and unknown bacterial tester strains. The thin white lines (arrows) represent antitoxin/DT precipitates ("ppt's") indicating bacterial DT production. Permission to show Rahul Gladwin's blog data (<http://www.rahulgladwin.com/noteblog/bacteriology/what-is-an-eleks-test.php>) is acknowledged.

For many years, the Schick test has been established to assess immunity to diphtheria toxin, although today it has been replaced in many regions by serologic tests for specific antibodies to diphtheria toxin. In the Schick test, 0.0124 microgram of diphtheria toxoid in 0.2 millilitre is injected intradermally at a control site and a small amount of diphtheria toxin (ca. 0.8 nanogram in 0.2 millilitre) is injected intradermally into the forearm (test site). Usually, after 48 and 96 hours respectively, readings are taken with non-specific skin reactions generally peaking by 48 hours. After 96 hours, an erythematous reaction with some possible necrosis at the test site indicates non-sufficient antitoxic immunity for neutralization of DT to occur (≤ 0.03 IU/millilitre). Inflammation at either test or control sites after 48 hours is indicative for a hypersensitivity reaction to the antigen preparation. In many instances, DT is only partially purified prior to inactivation with formaldehyde (see above) and as a result, preparations of toxoid may contain other corynebacterial products, which may elicit a (false positive) hypersensitivity reaction in some individuals.

Following initial isolation, *C. diphtheriae* may be identified as *mitis*, *intermedius*, or *gravis* biotype (see below) on the basis of physiological parameters including carbohydrate fermentation profiles and hemolysis on sheep blood agar plates. The toxigenicity of *C. diphtheriae* strains is determined by a variety of *in vitro* and *in vivo* tests. The most common *in vitro* assay for toxigenicity is the Elek immunodiffusion test (Fig. 3), which is based on the double diffusion of DT and antitoxin in an agar medium. A sterile, antitoxin-saturated filter paper strip is embedded in the culture medium and *C. diphtheriae* isolates are streak-inoculated at a 90° angle to the filter paper. The production of DT can be readily detected within 18 to 48 hours by the formation of a toxin-antitoxin precipitating band in the agar. Alternatively, many eukaryotic cell lines (e.g. African green monkey kidney or Chinese hamster ovary) are sensitive to DT, enabling *in vitro* tissue culture tests to be used for detection of toxin and DT-dependent ADP ribosylation of the cellular target protein, eukaryotic translation elongation factor 2 (EF2, see below). Several highly sensitive *in vivo* tests for DT have also been described (e.g. guinea pig challenge test, rabbit skin test). Clinical diagnosis depends upon culture-proven toxigenic *C. diphtheriae* infection of the skin, nose, or throat combined with clinical signs of nasopharyngeal diphtheria, i.e. dysphagia, sore throat, bloody nasal discharge, formation of pseudomembranes etc.

5. The diphtheria pathogen *Corynebacterium diphtheriae*

5.1 *C. diphtheriae* and diphtheria toxin (DT) production

Diphtheria is caused by *Corynebacterium diphtheriae*, in particular its pathovarieties or biotypes *gravis*, *intermedius* and *mitis* (reviewed by Murphy, 1996). The bacterial cells are Gram-positive, club-shaped, non-motile and non-capsulated (Fig. 1). Cultures grown in tissue or *in vitro* often contain typical cell wall spots that may affect the Gram reaction and are composed of characteristic polymetaphosphate inclusions; these are stainable with methylene-blue and appear as purple granules (Fig. 1). Although the three biotypes differ in colony morphology, growth performance and virulence, they all share the ability to secrete the lethal protein: diphtheria toxin (DT). Intriguingly, DT is specified for by *tox*, a gene carried on one of a family of related corynebacteriophages integrated into the host chromosome of *C. diphtheriae* (Fig. 4) (for review, see Bishai & Murphy 1988). That DT is encoded by the prophage gene was demonstrated when non-pathogenic strains of *C. diphtheriae* became lysogenically converted upon infection with a bacterial virus known as

beta phage (Freeman, 1957; Holmes & Barksdale, 1969). Moreover, mutant phages gave rise to nontoxic material that cross-reacted with diphtheria antitoxins, albeit being significantly shorter than full-length DT (Uchida et al., 1971).

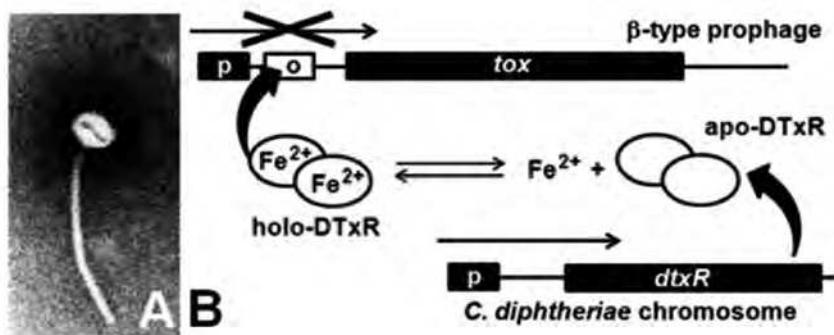


Fig. 4. Phage origin of *tox*, the structural gene encoding diphtheria toxin (DT), and model for *tox* gene regulation in the bacterial host *C. diphtheriae*. (A) Electron micrograph of the beta corynebacteriophage (for review, see Calendar, 1988) which carries the DT gene *tox* and upon infection and genomic integration converts non-toxigenic strains of *C. diphtheriae* into virulent ones. (B) Model of regulated *tox* gene expression by the repressor DTxR. Regulation of the phage *tox* gene depends on DTxR, a Fe^{2+} -binding and iron-responsive repressor dimer that is encoded by the *dtxR* gene on the *C. diphtheriae* genome (Tao, et al., 1994). This is why expression of the *tox* gene depends on the physiological state of the microbial host: under low iron conditions, Fe^{2+} ions dissociate from DTxR and liberate the *tox* gene operator from occupation by DTxR. This leads to *tox* gene derepression and DT can be expressed and secreted into the culture medium. In the presence of iron and upon binding Fe^{2+} ions, the holo-form of DTxR is recruited to the operator region upstream of *tox* and thereby prevents transcription of the DT gene by RNA polymerase to occur (D'Aquino et al., 2005). Upon depletion of Fe^{2+} from the medium, the holo-repressor complex (holo-DTxR) dissociates into its inactive apo-form (apo-DTxR) and the *tox* gene is relieved from transcriptional repression. The authors acknowledge permission by Professor Kenneth Todar, University of Wisconsin, USA, to reproduce a modified version of the electron micrograph (Todar, 2004) representing corynebacteriophage beta.

For optimal growth, some *C. diphtheriae* pathogens require thiamine or biotin and cultivation of most biotypes of *C. diphtheriae* depend on supplementation with nicotinic and pantothenic acids. In addition to being restricted to lysogenic bacteria, expression of the DT gene *tox* is controlled by an iron-responsive host repressor termed DTxR (Fig. 4) (Tao, et al., 1994). Thus, even though the *tox* gene is of viral origin, its regulation at the level of transcriptional repression/activation is coupled to the iron metabolism of the bacterial host (Tao, et al., 1994; D'Aquino et al., 2005). Therefore, optimal DT production is preferably achieved under conditions of low iron levels using culture medium that has been thoroughly deferrated. As for a physiological role of DT, the *tox* gene itself is not essential for the phage cycle and both synthesis and release of DT are not coupled to phage-induced lysis of *C. diphtheriae* cells. Also, it remains to be seen whether the lethal protein, which may account for ~5% of total protein expression in *C. diphtheriae*, benefits the bacterial life style in one way or another.

Nonetheless, by killing epithelial cells from infected pharyngeal niches, DT may contribute to colonization and virulence of the bacterial pathogen.

As early as 1887, Löffler described avirulent cells of *C. diphtheriae* from healthy individuals that were indistinguishable from virulent ones isolated from patients. It is now known that avirulent strains can be converted to virulent ones following infection with *tox* carrying corynebacteriophages *in vitro* and *in situ*. To this end, genetic drift of DT including horizontal gene transfer has not been described so that DT production appears to be confined to the three biotypes of *C. diphtheriae*. In addition to *C. diphtheriae*, other species of the genus *Corynebacterium* may occasionally cause infection of the nasopharynx and the skin. These include *C. ulcerans*, *C. pseudotuberculosis*, *C. pseudodiphtheriticum* and *C. xerosis* with the latter two being capable of producing pyrazinamidase, an intriguing enzyme which converts pyrazinamide (also used in prodrug treatment of *Mycobacterium tuberculosis*) to pyrazinoic acid (McClatchy et al., 1981). In veterinary medicine, *C. renale* and *C. kutscheri* are important pathogens which cause respectively, pyelonephritis in cattle and latent infections in mice.

5.2 DT, an A/B prototype toxin with ADP-ribosyltransferase activity

In sensitive species of humans, monkeys or rabbits, DT is extremely potent with as little as 100 nanograms of DT per kilogram of body weight being lethal. Protein structural analysis has revealed that DT, which is a 535 amino acid residue protein, is organized into individual protein domains with three distinct pathological functions: an N-terminal catalytic ADP-ribosyltransferase domain (i), a receptor binding domain for docking onto target cells (ii) and a transmembrane domain for subcellular delivery of the catalytic domain (iii) (Fig. 5) (Collier & Kandel, 1971; Gill & Pappenheimer, 1971; Gill & Dinius, 1971). Similar to the plant toxin ricin or *Pseudomonas* exotoxin A (ETA), DT is a prototype member of the classical A/B family of toxins (Lord & Roberts, 2005; Sandvig et al., 2005). In their secreted exo-forms, they mature by partial proteolysis into the N-terminal and C-terminal fragments A and B, respectively, which are held together by a disulfide bridge. While fragment B carries the receptor binding domain and the transmembrane motif (see above), segment A harbours the catalytic domain of DT.

Cell intoxication by DT is a multi-step process (Fig. 6) and involves (1) DT docking onto the cell surface receptor, (2) DT uptake and internalization by receptor-mediated endocytosis, (3) acidification of the endocytic vesicle by an ATP-driven proton pump, (4) uncoupling of fragment A from the A/B toxin and (5) delivery of the cytotoxic domain from the lumen of the endocytic vesicle into the cytosol (reviewed by Collier, 2001 and Ratts & Murphy, 2005). Next, by virtue of its catalytic activity, fragment A of DT targets the eukaryotic translation elongation factor 2 (EF2) for NAD⁺-dependent ADP-ribosylation (Fig. 6) (Collier & Cole, 1969; Pappenheimer, 1977). The resulting post-translational modification of EF2 by DT inhibits the essential elongation function of EF2 during *de novo* protein synthesis and eventually, leads to cell death (Fig. 6) (Van Ness et al., 1980; Sitikov et al., 1984). DT is an extremely potent agent and it has been demonstrated that subcellular import of a single molecule of its ADP-ribosylating domain toxin is sufficient for cell death induction. Studies on archaeal and eukaryal cells, which can both be killed by DT, demonstrate that the ADP-ribosylation reaction of DT is conserved and requires an exotic and highly modified histidine residue (Kimata & Kohno, 1994) in the EF2 target protein which is also known as

diphthamide (Fig. 7). Intriguingly, the EF2 analogues from the bacterial pathogens undergo no such diphthamide modification, which explains why *C. diphtheriae* cells are auto-immune and protected against their own ADP-ribosylase killer toxin (reviewed by Collier, 2001 and Ratts & Murphy, 2005).

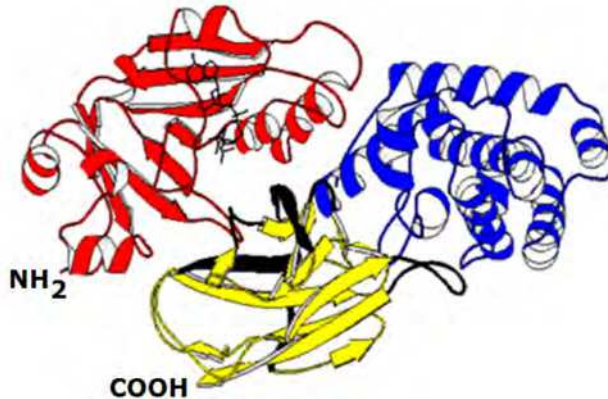


Fig. 5. MolScript-based ribbon diagram highlighting the modular domain organization of the DT monomer. The amino and carboxyl termini of full length DT are indicated (NH_2 and COOH , respectively). The domain in red represents the N-terminal ADP-ribosyltransferase catalytic centre which accounts for cytotoxic ADP-ribosylation and inactivation of the DT target protein EF2; the yellow motif illustrates the C-terminal binding domain important for cell surface attachment and receptor-mediated endocytosis of DT; the protein domain in blue is the transmembrane motif responsible for endosome insertion and subsequent subcellular release of the cytotoxic ADP-ribosylase domain. Permission by Professor Kenneth Todar, University of Wisconsin, USA, to reproduce a modified version of the illustration (Todar, 2004) is gratefully acknowledged.

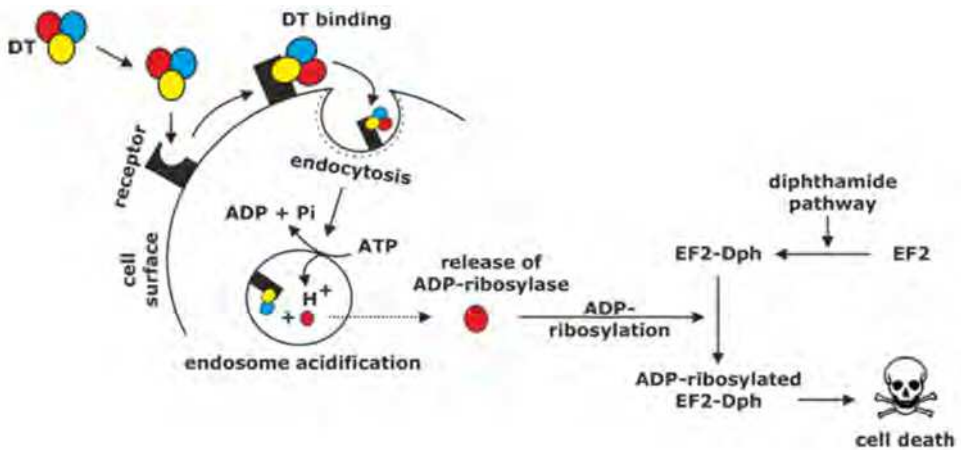


Fig. 6. Schematic diagram of eukaryotic target cell intoxication by DT. The toxin (with domain colour coding as introduced in Fig. 5) binds to its cell surface receptor by virtue of

its receptor binding (yellow) domain and is internalized by receptor-mediated endocytosis using clathrin-coated endosomes. Upon endosome acidification by a proton pump ATPase (pH ~5.1) located in the membrane of endocytic vesicles, the catalytic ADP-ribosylase domain (red) of DT becomes uncoupled from the receptor binding (yellow) and transmembrane (blue) domains. The catalytic domain is delivered to the cytosol and targets diphthamide-modified EF2 for ADP-ribosylation. This results in EF2 inactivation, inhibition of protein synthesis and eventually, death of the target cell. Scheme depiction has been inspired by previous DT reviews (for details see, Murphy, 1996; Collier, 2001; Todar 2004; Ratts & Murphy, 2005).

6. Diphthamide modification of eukaryotic translation elongation factor 2 (EF2)

6.1 Posttranslational biosynthesis of diphthamide on EF2

Diphthamide synthesis on EF2 operates through a complex pathway, which has been conserved among lower and higher eukaryotes (Chen et al., 1985; Moehring et al., 1984; Liu et al., 2004). In the budding yeast *Saccharomyces cerevisiae*, diphthamide biosynthesis requires at least five genes, *DPH1-DPH5* (Liu et al., 2004), two mammalian homologues of which (*DPH1/OVCA1* & *DPH3/KTI11*) are intriguingly involved in embryonic development and cell proliferation in rodents and humans (Fichtner & Schaffrath, 2002; Chen & Behringer, 2004; Fichtner et al., 2003; Liu & Leppa, 2003; Nobukuni et al., 2005; Liu et al., 2006). Though complex in nature, the diphthamide pathway has been shown to be molecularly dissectable. In *S. cerevisiae*, genetic screens selecting for resistance towards DT led to the isolation of diphthamide mutants that corresponded to five individual complementation groups (*dph1-dph5*) (Chen et al., 1985).

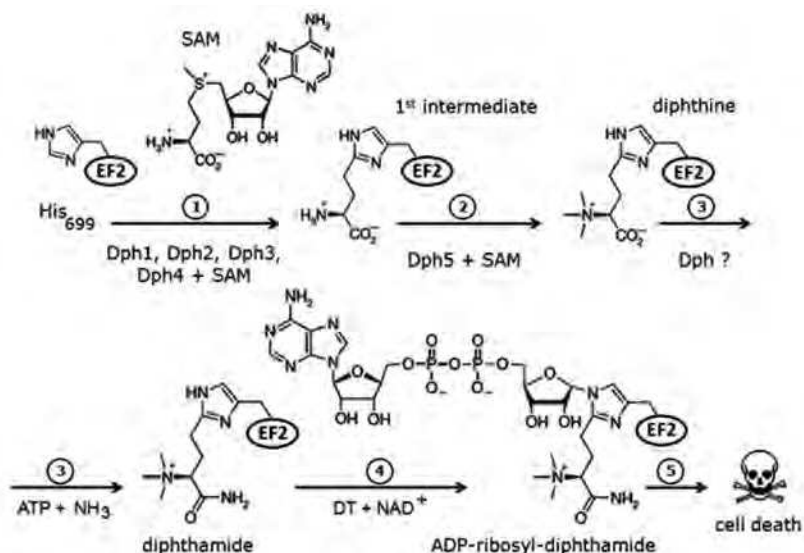


Fig. 7. Diphthamide modification on eukaryotic translation elongation factor 2 (EF2) in budding yeast operates through a multi-step pathway. The diphthamide pathway, modified

after Zhang et al. (2010), involves known and elusive (?) steps with the intermediates 2-(3-carboxyl-3-aminopropyl)-histidine and diphthine being generated. Abbreviations used: S-adenosylmethionine (SAM); adenosine triphosphate (ATP); nicotinamide adenine dinucleotide (NAD⁺). The pathway culminates in diphthamide-driven ADP-ribosylation of EF2 (ADP-ribosyl-diphthamide) by DT and other bacterial ADP-ribosylase toxins, including *Pseudomonas* exotoxin A or *Vibrio cholerae* cholix toxin (Zhang et al., 2008; Jørgensen et al., 2008) all of which induce cell death.

Diphthamide biosynthesis involves stepwise modifications starting with the transfer of the 3-amino-3-carboxypropyl (ACP) group from S-adenosylmethionine (SAM) to the C-2 position of the imidazole ring in the target histidine residue (EF2 His₆₀₀ from the archaeon *Pyrococcus horikoshii*; EF2 His₆₉₉ from budding yeast; EF2 His₇₁₅ in mammals) (Fig. 7). In yeast, this step depends on the diphthamide factors Dph1, Dph2, and Dph3 which all interact with each other (Fichtner et al., 2003; Liu et al., 2004; Bär et al., 2008) as well as Dph4, a J-protein (Webb et al., 2008) potentially chaperoning the Dph1-Dph3 complex. Eventually, Dph1-Dph4 action generates the first intermediate of the diphthamide modification pathway: 2-[3-carboxyl-3-aminopropyl]-histidine (Fig. 7) (Zhang et al., 2010). Next, the ammonium group of the intermediate undergoes trimethylation yielding the second intermediate diphthine (Fig. 7). This step is at least in part catalyzed by the protein methyltransferase Dph5 and requires three molecules of the methyl donor SAM (Fig. 7) (Chen & Bodley, 1988; Mattheakis et al., 1992). Finally, the carboxyl group of diphthine undergoes amidation (Fig. 7) in a process that is potentially catalyzed rather than spontaneous or non-enzymatic and likely to involve an as of yet unassigned ATP-dependent amidase (Fig. 7) (Liu et al., 2004). Once fully modified, the N-1 position of the diphthamide-imidazole ring (Fig. 7) is the site for NAD⁺-dependent ADP-ribosylation by DT. Intriguingly, other microbial ADP ribosylase toxins including *Pseudomonas* exotoxin A [ETA] (Zhang et al., 2008) and *Vibrio cholera* cholix toxin (Jørgensen et al., 2008) are known to target the diphthamide residue of EF2 in a highly similar, if not identical, manner. Eventually, ADP-ribosyl-diphthamide, the resulting terminal modification, irreversibly inactivates the translation elongation function of EF2 (Fig. 7) (Sitikov et al., 1984).

As for the elusive and terminal amidation step, no DT resistant yeast mutants have been identified to date, probably because diphthine is a substrate (though poor) for ADP-ribosylating toxins. Provided terminal amidation was an enzymatically catalysed process rather than a spontaneous one (see above), amidase-deficient mutants may still display DT sensitivity, which is why the amidase in question may have repeatedly escaped identification in the above screens for DT resistance. It will be interesting to see whether this also holds true for screens involving EF2 inhibitors or antagonists that are not related to DT but share with DT a common requirement for diphthamide modification of EF2. This is of particular interest in the light of recent evidence that *dph1*, *dph2*, *dph3*, *dph4* and *dph5* deletion mutants from yeast not only are protected against DT (Fig. 8) but are also all resistant to growth inhibition by sordarin (Fig. 8) (Bär et al., 2008; Botet et al., 2008). The latter is an ascomycetous glycoside (Hauser & Sigg, 1971) whose antifungal activity obviously depends on diphthamide, but that operates by selectively blocking the EF2-ribosome complex rather than inhibiting EF2 by ADP-ribosylation (Dominguez et al., 1999).

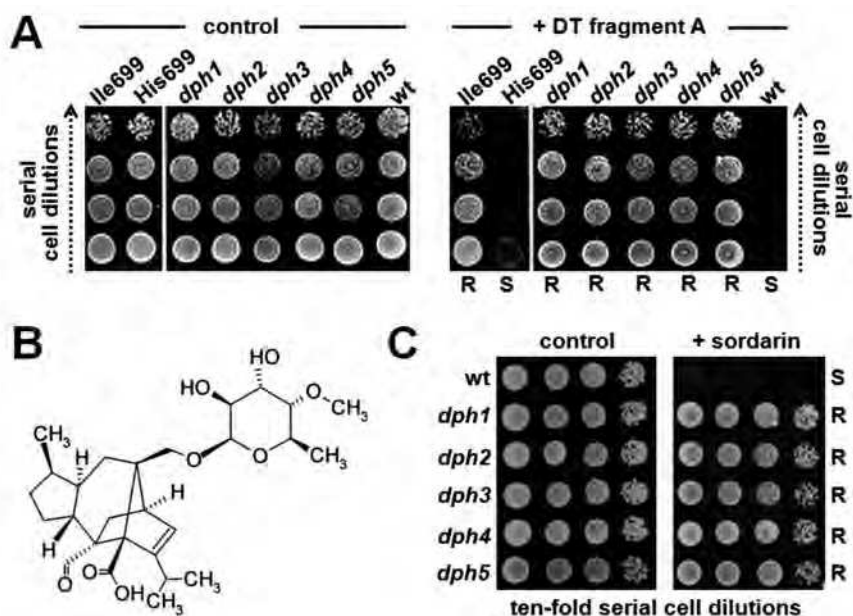


Fig. 8. Diphthamide modification is essentially required for growth inhibition of yeast cells by DT and by the antifungal sordarin. (A) DT resistance due to defects in diphthamide synthesis on EF2. Yeast cells with an EF2 diphthamide target residue substitution (His699Ile) (Kimata & Kohno, 1994) and diphthamide mutants (*dph1*, *dph2*, *dph3*, *dph4* and *dph5*) (Chen et al., 1985) resist (R) against conditional expression of the lethal DT fragment A while wild-type cells (wt; His699) remain sensitive (S) to the ADP-ribosylase and are killed (right panel). Empty vector control (left panel) shows the growth control in the absence of DT. (B) Chemical formula of sordarin, an EF2-specific antifungal and ribosome inhibitor. (C) Like DT, sordarin action requires diphthamide synthesis on EF2. Wild-type (wt) parental strain W303 and its diphthamide mutants (*dph1*-*dph5*) were cultivated in the absence (control) or presence of the antifungal (+ sordarin). A resistant (R) cell response is distinguished from sensitivity (S).

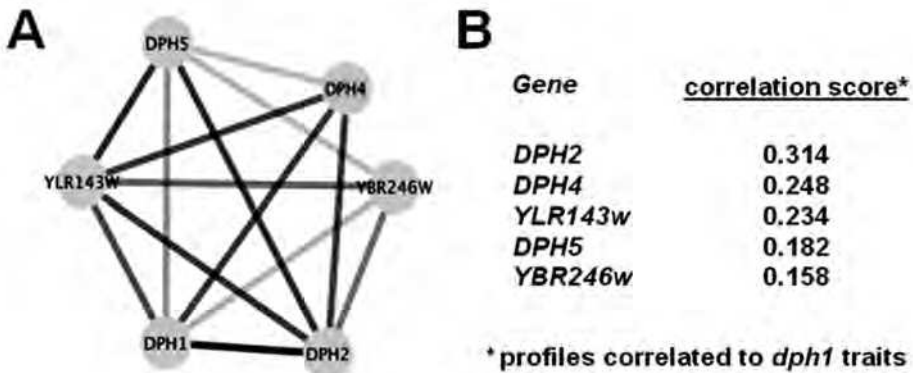


Fig. 9. Based on synthetic genetic array (SGA) analysis, yeast Open Reading Frames (ORFs) *YLR143w* and *YBR246w* represent loci that are potentially related to the *DPH* genes and the diphthamide pathway. (A) Genetic interaction data (Baryshnikova et al., 2010) indicating relatedness between ORFs *YLR143w* and *YBR246w* and diphthamide synthesis genes *DPH1*, *DPH2*, *DPH4* and *DPH5*. (B) Phenotypic clustering (Carette et al., 2009) and phenotypic scores, in relation to diphthamide mutant *dph1*, between *YBR246w* and *DPH5* genes as well as *YLR143w* and *DPH4* suggest both ORFs to be related to *DPH1* and to the diphthamide modification pathway.

In an effort to further analyze the relationships among individual components of the diphthamide pathway, we found that the Dph1, Dph2 and Dph3 proteins form a protein complex whose assembly is crucial for diphthamide formation and consequently for ADP-ribosylation of EF2 by DT (Fig. 10). Strikingly, the *DPH3* gene from budding yeast was shown to be allelic with the locus Killer Toxin Insensitive 11 (*KTI11*) (Butler et al., 1994). *KTI11* was shown to be required for the *Kluyveromyces lactis* tRNAse toxin zymocin to kill other yeast species including *S. cerevisiae* (Fichtner & Schaffrath, 2002). In particular, the Kti11/Dph3 gene product was shown to be involved in a tRNA modification pathway that is essential for the tRNAse activity of zymocin to cleave target tRNAs and cause yeast cell death by way of tRNA depletion (Huang et al., 2005; Lu et al., 2006; Jablonowski & Schaffrath, 2007; Jablonowski et al., 2006; Kheir et al., 2011). In addition to interacting with Dph1, Dph2 and EF2 (Fichtner et al., 2003; Bär et al., 2008), Dph3/Kti11 was furthermore shown to communicate with other proteins (Kti13: Zabel et al., 2008) or protein complexes (Rvs161•Rvs167; Elongator complex: Fichtner et al., 2003; Krogan et al., 2006) suggesting multiple roles for Kti11/Dph3 in processes not necessarily limited to the diphthamide modification pathway. In support of such versatility, DelGIP1 (the human homologue of yeast Kti11/Dph3) interacts with deafness locus-associated guanine nucleotide exchange factor (DelGEF) and the DelGIP1•DelGEF protein complex affects exocyst-dependent secretion of proteoglycans (Sjölander et al., 2002, 2004). Also, our group was able to show that conditional phenotypes and stress-inducible growth defects of a yeast mutant with a single *KTI11/DPH3* gene deletion were more severe and pronounced in relation to rather mild defects of yeast mutants lacking *DPH1* or *DPH2* gene function (Bär et al., 2008), supporting its role in a wider range of cellular functions in yeast. Atomic absorption

spectroscopy has recently shown that the Kti11/Dph3 protein folds into a closed compact and globular protein structure with its C-terminal alpha-helix protruding outward (Sun et al., 2005). Moreover, the protein co-ordinates a zinc ion via a $Zn(Cys)_4$ binding module that is highly conserved among Kti11/Dph3 homologues from plants, animals and humans (Proudfoot et al., 2008). Presumably, it is this motif that is engaged in the putative electron-carrier activity recently proposed for Kti11/Dph3 by Proudfoot et al. (2008). In line with this notion, both single and multiple Cys substitutions of the four critical residues in the potential $Zn(Cys)_4$ binding module cause inactivation of the Kti11/Dph3 variants and traits including resistance to growth inhibition by DT, sordarin and zymocin that are identical to the phenotypes of null-mutants lacking *KTI11/DPH3* gene function (Fichtner & Schaffrath, 2002; Bär et al., 2008).

Strikingly, the multi-step pathway for diphthamide formation and EF2 modification (Fig. 7) has been conserved from lower to higher eukaryotes. Among the five budding yeast diphthamide genes (*DPH1*, *DPH2*, *DPH3*, *DPH4* and *DPH5*) (Fig. 7), there are two mammalian homologues (*DPH1/OVCA1* and *DPH3/KTI11*) that are required for cell proliferation, tumourigenesis and neuronal development in mice and human cells. As a result, defects in *DPH3/KTI11* are associated with neurodegeneration in mice (Liu et al., 2006) and mutations in *OVCA1/DPH1* have identified a tumour suppressor role for this diphthamide-related gene product in the context of ovarian cancer (Chen & Behringer, 2004). In an effort to further study diphthamide function and the interrelation between components of the diphthamide pathway, we found by co-immune precipitation and tandem affinity purification protocols that the Dph1, Dph2 and Dph3 factors form a protein complex, assembly of which is crucial for EF2 ADP-ribosylation by DT (Fichtner et al., 2003; Bär et al., 2008). Moreover, we and others discovered that the Dph1-Dph5 proteins are all required for the cytotoxic activity of sordarin (Bär et al., 2008; Botet et al., 2008), another EF2-related antifungal and translation inhibitor (Justice et al., 1998).

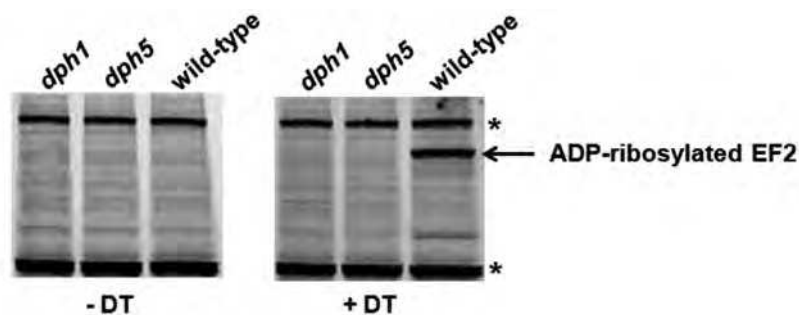


Fig. 10. Yeast diphthamide modification mutants evade ADP-ribosylation of EF2 by DT. Shown are EF2 ADP ribosylation assays on total protein extracts from the indicated yeast strain backgrounds in the absence (left panel) and presence (right panel) of recombinant DT. When using biotin-labeled NAD^+ as donor for the *in vitro* ADP ribosylation assay, wild-type strains display EF2 ADP-ribosylation acceptor activity (indicated by the arrow) whereas diphthamide mutants *dph1* and *dph5* fail to do so. The asterisks denote unspecific signals in proteins irrespective of DT treatment and/or strain backgrounds tested.

In a search for new diphthamide-related factors, two novel and uncharacterized open reading frames (ORFs), YBR246w and YLR143w, have been identified recently as new potential components for diphthamide biosynthesis using genetic screens in human and yeast cells (Botet et al., 2008; Carette et al., 2009). Based on synthetic genetic interaction data deposited at the genetic interaction database (GID; University of Toronto, Canada) (Baryshnikova et al., 2010) and the significance of the phenotypic correlation scores, both budding yeast ORFs are predicted to have EF2-related functions (Fig. 9). In addition, possible effector roles of YBR246w and YLR143w for EF2 specific antifungals including sordarin are becoming evident: when deleted, these new loci not only affect the communication between Dph5 and EF2 but also phenocopy traits (including sordarin resistance) that are typical of *dph1*, *dph2*, *dph3*, *dph4* and *dph5* mutants from yeast (Bär et al., 2008; Botet et al., 2008; Carette et al., 2009). Although being aware that the sordarin phenotype may also be ascribable to defects in EF2-unrelated genes that are required for binding and/or import of the deadly antifungal (Botet et al., 2008), we consider these ORFs to be candidate diphthamide biosynthesis genes. In support of this notion, preliminary data based on *in vitro* EF2 modification assays demonstrate that inactivation of YBR246w and YLR143w eliminates the ADP-ribosylation acceptor activity of EF2 in the presence of DT. Since this is a trait that is specific to the *bona fide* diphthamide synthesis defect of *dph1*, *dph2*, *dph3*, *dph4* and *dph5* mutants (Liu & Leplla, 2003; Liu et al., 2004) (Fig. 10), YBR246w or YLR184w deletion may cause a diphthamide defect, too, which abolishes DT-dependent ADP-ribosylation of EF2.

To sum up, diphthamide incorporation of EF2 is not only pathologically relevant for ADP-ribosylation by DT but also crucial for toxicity of the antifungal sordarin. Physiologically, the diphthamide pathway appears to be important for mRNA translation as well as proper cell proliferation and neural development in eukaryal cells. Surprisingly, our data imply that formation of diphthamide is genetically more complex than originally anticipated (Chen et al., 1985; Liu et al., 2004) and that the pathway may comprise more gene products than the five Dph1-Dph5 members known to date (Carette et al., 2009). For future work, it will be significant to define the roles of new diphthamide candidates and how they may relate to or communicate with the other known pathway members.

6.2 Biological significance for diphthamide modification of EF2

Diphthamide on EF2 is the target for bacterial ADP-ribosylase toxins (DT; ETA; cholix) and also affects toxicity of sordarin and ricin, a ribosome inhibiting protein toxin from plants (Gupta et al. 2008). Although this emphasizes its varied pathological relevance, the physiological significance of diphthamide remains enigmatic and elusive. Nonetheless, the evolutionary conservation of the diphthamide pathway among eukaryotes strongly suggests that diphthamide will be important in processes including mRNA translation. In support of this notion, evidence from research groups including our own has shown that diphthamide defects increase translational frame-shifting (Ortiz et al., 2006; Bär et al., 2008). Moreover, homologues of diphthamide synthesis genes (*DPH1/OVCA1* and *DPH3/KTI11*) affect the proliferation and development of mammalian cells, which is why inactivation of *DPH3/KTI11* is associated with tRNA modification defects and neurodegeneration and mutations in *DPH1/OVCA1* revealed a tumour suppressor role for this diphthamide synthesis gene in ovarian cancer (Chen & Behringer, 2004; Nobukuni et al., 2005; Huang et al., 2005; Liu et al., 2006; Kim et al., 2010).

Whether or not this implies structural or regulatory roles for diphthamide in mRNA translation remains to be seen. The latter, however, is intriguing with the emergence of a cellular ADP-ribosyltransferase that resembles the diphthamide-dependent ADP-ribosylation reaction by DT (Lee & Iglewski, 1984; Jäger et al., 2011). As a result, diphthamide may be envisioned to be used as an on/off switch for endogenous ADP-ribosylation of EF2 and control of mRNA translation and protein synthesis. Irrespective of unclear physiological functions, recent genetic data imply that the diphthamide pathway is more complex than originally anticipated and likely to comprise further components, in addition to Dph1-Dph5 (Carette et al., 2009). For future research, it will be therefore crucial to define the identity of new diphthamide synthesis candidates and provide insights into how they communicate with known members of the pathway.

7. Engineering DT chimera for use in cell-specific proliferation control

Protein engineering is a new and rapidly developing area within the field of molecular biology; it brings together recombinant DNA methodologies and solid phase DNA synthesis in the design and construction of chimeric genes whose products have unique properties. Through a combination of protein engineering and DT structure-function studies, it has been possible to genetically substitute the native DT receptor-binding domain B (Figs. 5 and 6) with a variety of polypeptide hormones and cytokines (e.g. α -melanocyte-stimulating hormone [α -MSH], interleukin [IL] 2, IL-4, IL-6, IL-7, epidermal growth factor, etc) (Foss, 2001; Kreitman, 2006, 2009). The resulting fusion toxins or chimera combine the receptor-binding specificity of the cytokine with the catalytic ADP-ribosylase domain of DT. In each instance, the chimeric proteins have unique properties and selectively attack only those target cells that bear the appropriate target cell receptor on the cell surface. One of these engineered fusion toxins, DAB389IL-2 (ONTAK) (Le Maistre et al., 1992), has been evaluated in clinical trials for the treatment of human lymphomas, in which cells with high affinity IL-2 receptors play a major role in pathogenesis. Administration of ONTAK has been shown to be well tolerated, safe and to induce durable remission from disease in the absence of undesired side effects. Moreover, ONTAK and its predecessor, DAB486IL-2 (Le Maistre et al., 1992) have demonstrated activity in a variety of diseases, including cutaneous T cell lymphoma (CTCL), psoriasis, rheumatoid arthritis and HIV infection. Hence, DT-based fusion toxins are important biological agents for the treatment of certain tumours or disorders in which specific cell surface receptors can be selectively targeted (Hesketh et al., 1993; Van der Spek et al., 1994; Foss, 2001; Kreitman, 2006, 2009) and it is likely that such DT chimera will be providing further and important new biological tools for selected cell targeting and DT-dependent inactivation of protein biosynthesis, a fundamental biological process with key roles for cell cycling and cancer formation (White-Gilbertson et al., 2009).

8. Conclusion

Diphtheria represents one of the best studied bacterial diseases of humans with its etiology, mode of transmission, pathogenic mechanism and molecular basis of DT structure and function being clearly established. Consequently, highly effective methods for treatment and prevention of diphtheria have been developed and many contributions to the fields of medical microbiology, immunology and molecular biology as well as to our understanding of host-bacterial interactions and pathogenesis have been made possible by studying

diphtheria and DT. Diphtheria is caused by *C. diphtheriae*, pathovar. *gravis*, *intermedius* and *mitis*, three biotypes that differ in virulence and growth performance but share the ability to secrete the lethal ADP ribosylase toxin DT, the protein product of a lysogenic phage gene. The DT gene *tox* is under control of an iron-responsive repressor (DtxR) so that DT production is limited under conditions of low iron levels and to lysogenic bacteria only. DT is a typical A/B toxin containing two fragments that are proteolytically processed from a single precursor and held together by a disulfide bridge. The A fragment is catalytically active and the B fragment promotes receptor-mediated endocytosis of DT. Upon import, the A subunit is cleaved-off from the B fragment and gets released into the cytoplasm. Here, DT unfolds its toxicity and ADP-ribosylates eukaryotic translation elongation factor 2 (EF2). ADP-ribosylation of EF2 by DT is irreversible, eventually inhibiting mRNA translation and protein synthesis and inducing the death of the target cell. Studies from archaeal and eukaryal target cells demonstrate that the ADP-ribosylase activity of DT requires diphthamide, a highly modified histidine residue in EF2. Intriguingly, the EF2 analogues from *Corynebacteria* lack diphthamide, which explains why the DT producers are immune to their own toxin.

Strikingly, diphthamide formation on EF2 operates through a multi-step pathway that is conserved among archaea and eukaryotes. In the yeast *S. cerevisiae*, it comprises at least five different genes, *DPH1-DPH5*, of which two mammalian homologues (*DPH1/OVCA1* & *DPH3/KTI11*) are required for cell proliferation, tumorigenesis and neuronal development. As a result, defects in *DPH3/KTI11* are associated with neurodegeneration and mutations in *OVCA1/DPH1* have identified a tumour suppressor role for the diphthamide-related product in ovarian cancer. In an effort to further study the diphthamide pathway, we found that the Dph1, Dph2 and Dph3 factors form a protein complex, assembly of which is crucial for EF2 ADP-ribosylation by DT. Moreover, all five Dph1-Dph5 proteins are required for the cytotoxic activity of sordarin, another EF2-related inhibitor. In a search for novel diphthamide-related genes from yeast by use of synthetic genetic array (SGA) analysis and the genetic interaction database (GID), we identified two open reading frames (ORFs: YBR246W; YLR143w) previously implicated in antifungal activity of DT and sordarin. In line with predicted EF2 roles, deletion mutants lacking YBR246W or YLR184w are resistant towards doses of sordarin that are lethal to wild-type yeast cells. Moreover, EF2 modification assays in the presence of DT demonstrate that protein extracts from the deletion strains lack ADP-ribosylation acceptor activity of EF2. This suggests that YBR246W or YLR184w inactivation may have caused a diphthamide biosynthetic defect, which abrogates DT-dependent ADP-ribosylation of EF2. In sum, diphthamide incorporation of EF2 is not only relevant for ADP-ribosylation by DT but also crucial for toxicity of the antifungal sordarin. Physiologically, the diphthamide pathway appears to be important for mRNA translation as well as proper cell proliferation and neural development in eukaryal cells. Surprisingly, our data imply that formation of diphthamide is genetically more complex than originally anticipated and that the pathway may comprise more than the five Dph1-Dph5 gene products known to date.

Finally, the study of diphtheria toxin structure/function relationships has clearly shown DT toxin to be a three-domain protein with individual roles for receptor binding, endocytosis and catalysis (i.e. NAD⁺-dependent ADP-ribosylation). Through protein engineering, a rapidly developing area within the field of molecular biology that brings together recombinant DNA methodologies and solid phase DNA synthesis, the design of diphtheria

fusion toxin genes has been feasible whose products have unique properties. Thus, it has been possible to genetically substitute the native diphtheria toxin receptor-binding domain with a variety of polypeptide hormones and cytokines so that the resulting fusion toxins combine the receptor-binding specificity of the cytokine with the ADP ribosylase activity of DT. The fusion toxins can selectively intoxicate only those cells which bear the appropriate targeted receptor. It is likely that such DT-based fusion toxins will be important new biological agents for the treatment of tumours/disorders in which specific cell surface receptors may need to be targeted.

9. Acknowledgment

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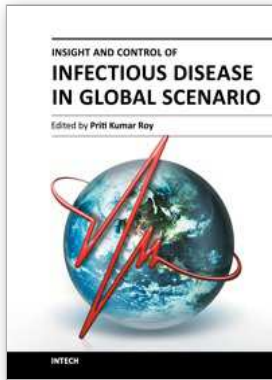
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This book is projected as a preliminary manuscript in Infectious Disease. It is undertaken to cover the foremost basic features of the articles. Infectious Disease and analogous phenomenon have been one of the main imperative postwar accomplishments in the world. The book expects to provide its reader, who does not make believe to be a proficient mathematician, an extensive preamble to the field of infectious disease. It may immeasurably assist the Scientists and Research Scholars for continuing their investigate workings on this discipline. Numerous productive and precise illustrated descriptions with a number of analyses have been included. The book offers a smooth and continuing evolution from the principally disease oriented lessons to a logical advance, providing the researchers with a compact groundwork for upcoming studies in this subject.

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